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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁴ : C12Q 1/68, C07H 21/00		A1	(11) International Publication Number: WO 88/ 06633 (43) International Publication Date: 7 September 1988 (07.09.88)
(21) International Application Number: PCT/US88/00550		San Diego, CA 92123 (US).	
(22) International Filing Date: 2 March 1988 (02.03.88)		(74) Agents: KENNEY, J., Ernest; 625 Slaters Lane - Fourth Floor, Alexander, VA 22314 (US) et al.	
(31) Priority Application Number: 020,866		(81) Designated States: AU, DK, FI, JP, KR, NO, US.	
(32) Priority Date: 2 March 1987 (02.03.87)		Published <i>With international search report.</i> <i>With amended claims.</i>	
(33) Priority Country: US			
(60) Parent Application or Grant (63) Related by Continuation US Filed on 020,866 (CIP) 2 March 1987 (02.03.87)			
(71)(72) Applicants and Inventors: ARNOLD, Lyle, John, Jr. [US/US]; 5439 Noah Way, San Diego, CA 92117 (US). NELSON, Norman, Charles [US/US]; 3639 Marlesta Drive, San Diego, CA 92111 (US). REYNOLDS, Mark, Alan [US/US]; 3115-G Evening Way, La Jolla, CA 92037 (US). WALDROP, Alexander, A., III [US/US]; 9249 Village Glen Drive,			

(54) Title: POLYCATIONIC SUPPORTS FOR NUCLEIC ACID PURIFICATION, SEPARATION AND HYBRIDI-ZATION

(57) Abstract

Described herein is the use of polycationic solid supports in the purification of nucleic acids from solutions containing contaminants. The nucleic acids non-covalently bind to the support without significant binding of contaminants permitting their separation from the contaminants. The bound nucleic acids can be recovered from the support. Also described is the use of the supports as a means to separate polynucleotides and hybrids thereof with a nucleotide probe from unhybridized probe. Assays for target nucleotide sequences are described which employ this separation procedure.

ATTORNEY DOCKET NUMBER.: 1803-337
SERIAL NUMBER.: 09/756,743
REFERENCE: A93

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POLYCATIONIC SUPPORTS FOR NUCLEIC ACID
PURIFICATION, SEPARATION AND HYBRIDIZATION

5 1. Field of the Invention

This invention relates to polycationic supports and their use for purifying nucleic acids, immobilizing nucleic acids for hybridization procedures, and selectively separating relatively large hybridized polynucleotides from solution without significantly removing unhybridized smaller polynucleotide or oligonucleotide probes.

10 2. Description of the Prior Art

15 In recent years, the desire to purify polynucleotides and to conveniently carry-out hybridization reactions to detect specific nucleotide sequences in polynucleotides has increased dramatically. For this reason, numerous methods have been developed 20 for separating polynucleotides and their hybrids and for detecting specific target nucleotide sequences in polynucleotides employing polynucleotide or oligonucleotide probes.

25 In the area of polynucleotide purification, procedures are employed to either purify polynucleotides prior to hybridization or to remove biological contaminants which might interfere with various biochemical procedures. Historically, different methods have been employed such as phenol extraction, ethanol precipitation, gel electrophoresis, gel permeation

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Shih & Martin, Biochemistry, 13:3411-3418 [1974]; Enea & Zinder, Science, 190:584-586 [1975]; Shih & Khoury, Biochemistry 15:487-493, [1976]; Longacre & Mach, J. Biol. Chem. 253:7500-7507 [1978]; Goldberg et al., Methods in Enzymol., 68:206-242 [1979]; Kreig et al., Analytical Biochem., 134:288-294 [1983]; and Gautreau, et al., Analytical Biochem., 134:320-324 [1983]).

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More recently, high pressure liquid chromatography has been used for preparative scale purifications of single and double stranded polynucleotides. Anion exchange columns employed in this method include RPC-5 (Pearson et al., Biochem. Biophys. Acta., 228, 770-774, [1971]), other coated supports incorporating Kel-F (Shum and Crothers, Nucl. Acids Res., 5, 2297-2311, [1978]) or siliconized glass beads (Narihara et al., J. Chromatog., 236, 513-518, [1982]) and diethylamino-ethyl-derivatized silica such as Nucleogen^{1/} (Colpan and Riesner, J. Chromatog., 296, 339-353, [1984]; Hecker et al., J. Chromatog., 326, 251-261, [1985]; and Muller, Eur. J. Biochem., 155, 203-212, [1986]).

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In addition, other recent methods of purification include both the selective adsorption of polynucleotides to surfaces of specialized supports (Johnson et al., Biotechniques, 4, 64-70 [1986], Guenther et al., Fed. Proc., 44, 1622 (abs. 7086) [1985] and immobilization of polynucleotides by complementary nucleic acid sequences (Nanibhushan & Crothers, Eur. Pat. App. No. 130, 523 [1985]; Ruth et al., Fed. Proc. 44, 1622 (abs. 7088), 1985; Blakesley and Thompson, Int. Pat. App. No. PCT/US84/00508 [1985]).

1/ Nucleogen is a registered trademark of Macherey-Nagel, Duren, West Germany.

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The purification methods mentioned above, however, continue to suffer from one or more of the following limitations, particularly when adapting them to a clinical environment. These limitations include:
5 They are time consuming or laborious; they do not quantitatively recover the desired polynucleotides; they are not compatible with clinical samples; or they cannot be easily interfaced to automated systems.

In addition to purifying polynucleotides, solid
10 supports have been used for separating hybrids of a polynucleotide and a nucleotide probe from unhybridized polynucleotide or oligonucleotide probes. Hybridization of polynucleotides in conjunction with appropriately labeled polymeric nucleotide probes has long been recognized as a specific and sensitive method for detecting unique or misexpressed polynucleotide target sequences. This ability permits the detection of
15 infectious organisms such as bacteria, viruses, parasites and fungi; genetic diseases such as sickle cell anemia; and various cancers. However, the inability to rapidly and conveniently separate hybridized from unhybridized polynucleotides has limited the use of hybridization procedures in clinical diagnosis and the research environment.
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25 For more than twenty years, researchers have been working to develop methods to identify the presence of "target" polynucleotides employing hybridization. These methods have largely been based upon separating polynucleotide hybrids from unhybridized polynucleotide or oligonucleotide probe molecules. In 1963 and 1964,
30 Nygaard & Hall (Biochem. Biophys. Res. Commun., 12, 98 [1963]; J. Mol. Biol., 9, 125 [1964] reported a method for separating DNA/RNA hybrids by filtration through

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nitrocellulose. These researchers found that single and double stranded RNA did not stick to nitrocellulose, but that single stranded DNA or DNA complexed to RNA did stick to nitrocellulose. Thus, a reaction between radiolabeled RNA and unlabeled DNA could be monitored by measuring the amount of radioactivity caught on the filter.

This procedure was followed by one described by Gillespie and Spiegelman, (J. Mol. Biol., 12, 829 [1965]) wherein single stranded DNA was immobilized on nitrocellulose filters and hybridized with a solution containing radiolabeled RNA. After removal of excess RNA, the degree of hybridization was determined by the amount of radiolabel immobilized on the filter.

The following year, Denhardt (Biochem. Biophys. Res. Commun., 23, 641 [1966] reported the development of a similar system whereby single-stranded DNA was immobilized to nitrocellulose, the filter "capped" to reduce non-specific background, and the immobilized DNA hybridized with a solution containing an appropriate radiolabeled DNA probe. This has remained as one of the more preferred hybridization procedures to date. This procedure, however, is laborious and time consuming; normally requiring more than a day to complete.

In addition to nitrocellulose, hydroxyapatite has been used to separate single stranded and double stranded polynucleotide species. Britten and Kohne, for example, (Carnegie Inst. Washington Yearbook, 65, 78 [1966]) used hydroxyapatite to separate [³²P]-labeled E. coli DNA fragments which were hybridized from E. coli DNA fragments which were not hybridized. These methods were further refined by

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Brenner et al. (Anal. Biochem., 28, 447 [1969]), to carry out separations in test tubes.

Other procedures have been developed in an effort to further improve immobilization of DNA and RNA for hybridization reactions. These include fixation of DNA and RNA to cyanuric chloride-activated paper (Hanger et al., Biochem. Biophys. Acta, 653, 344 [1981]) and aryl diazonium cellulose papers (Seed, Nucl. Acids Res., 10, 1799 [1982]).

Procedures have also been described for visualizing non-isotopically labeled biotinylated probes hybridized to DNA or RNA immobilized on nitrocellulose (Leary et al., Proc. Natl. Acad. Sci. U.S.A., 80, 4045 [1983]). This procedure, however, is even more laborious than previous protocols employing radiolabels since it requires additional "capping" and washing steps.

In an effort to improve the detection of "target" polynucleotide employing hybridization, numerous procedures have been developed employing a double (sandwich) hybridization. Usually an immobilized polynucleotide is employed to find the target polynucleotide followed by hybridization with a second polynucleotide which is labelled (Dunn & Sambrook, Methods Enzymol., 65, 468-478 [1980]; Palva, Journal Clin. Micro., 18, 92-100 [1983]; Virtanen et al., Lancet, 381-383 [1983]; Ranki et al., Gene, 21, 77-85 [1983]; Ranki & Soderland, U.S.P.N. 4,486,539 [1984]; Polsky-Cynkin et al., Clin. Chem., 31, 1438-1443 [1985]; Ranki & Soderlund U.S.P.N. 4,563,419 [1986]).

In one application of the sandwich assay procedure, both hybridization reactions are carried out in solution followed by separation on immobilized streptavidin which separates the entire polynucleotide

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sandwich using a biotin labeled probe (Hansen & Jones, E.P.O. 0 139 489 [1985]).

5 The hybridization systems described above still lack the ease of handling and speed to make hybridization assay procedures widely useful in a clinical environment. They are generally time consuming, laborious and are lacking in sensitivity. In addition, many of them are incompatible with agents contained in biological samples and they cannot be
10 readily automated.

3. Definitions

As used in this disclosure, the following terms are defined as:

15 nucleotide: a subunit of a nucleic acid consisting of a phosphate group, a 5 carbon sugar and a nitrogen containing base. In RNA the 5 carbon sugar is ribose. In DNA, it is 2-deoxyribose.

20 nucleotide multimer: a chain of nucleotides linked by phosphodiester bonds.

25 oligonucleotide: a nucleotide multimer generally about 10 to about 100 nucleotides, but which may be 200 or more nucleotides in length. They are usually synthesized from nucleotide monomers or obtained by enzymatic means.

30 polynucleotide: a nucleotide multimer generally about 100 nucleotides or more in length.

nucleotide probe: a nucleotide multimer having a nucleotide sequence complementary with a target nucleic acid sequence contained within a second nucleotide multimer, usually a

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polynucleotide, having diagnostic significance. Usually the probe is selected to be perfectly complementary to the target sequence. However, in some cases it may be adequate or even desirable that one or more nucleotides in the probe not be complementary to the corresponding base in the target sequence. A nucleotide probe is also usually a smaller multimer than the multimer containing the target sequence. Typically it is an oligonucleotide, but may be a polynucleotide and is usually labeled with a chemical substituent which permits its detection, for example, by radiometric colorimetric, fluorometric, chemiluminescence or other suitable techniques.

separation solution: A solution having a composition which permits immobilization of a nucleotide multimer, usually a polynucleotide, or a hybrid thereof to a cationic solid support as described herein without binding contaminants including in certain cases, smaller polynucleotides and oligonucleotides. When a polynucleotide hybridized with a probe is being immobilized, the separation solution has the additional property of inhibiting binding of the nucleotide probe to the support.

wash solution: A solution with a composition to permit removal of excess nucleotide probe or contaminants without significantly removing a desired nucleotide multimer, usually a polynucleotide or a desired polynucleotide hybrid, from the surface of a cationic solid

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support as described herein. The composition of a wash solution may, in some cases, be the same as a separation solution.

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elution solution: A solution designed to liberate a nucleotide multimer such as a polynucleotide, a hybridized polynucleotide, or a labeled nucleotide probe, or a specific label from the surface of a cationic solid support, the specific liberation done being dependent upon the desired kind of recovery from the solid support that is desired.

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hybridization solution: A solution designed to permit a desired hybridization to occur. The desired hybridization is typically between a polynucleotide and a probe for a target sequence. Therefore, when hybridization occurs with a polynucleotide previously immobilized on a cationic solid support, this solution has the additional property of minimizing the non-specific binding of the nucleotide probe to the support.

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4. Summary of the Invention

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This invention is based upon the discovery that polycationic solid supports can be used to selectively adsorb nucleotide multimers according to their size, larger multimers being more tightly bound to the support than smaller ones. The binding interaction is believed to be based, at least in part, on the ionic attractive forces between a positively charged support and the negatively charged sugar phosphate backbone of the nucleotide multimer. These properties may be employed in batch procedures to both rapidly purify

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polynucleotide and separate hybrids thereof from complex solutions.

One method according to the invention permits purification of nucleotide multimers in solutions containing various constituents of the organism from which the multimers are obtained including multimers of lower molecular weight, by adsorbing the desired multimers to the polycationic support. In the case of clinical samples, the nucleotide multimers are also removed from other constituents of the body fluids and tissues from which the multimers are to be separated. After separation of the solid support from the solution, the multimers may be eluted from the support.

In another method according to the invention useful in hybridization procedures to detect relatively long polynucleotides with shorter, labeled nucleotide probes, the polycationic support can be used to separate polynucleotides, and hybrids thereof with the probe, from the unhybridized probe. In a presently preferred embodiment of this method, the probe is added to a solution containing polynucleotides and hybridization allowed to occur prior to immobilization. After hybridization with the probe, the solution is contacted with the support to adsorb the polynucleotides, including any hybrid thereof with the probe. In a presently less preferred embodiment, the polycationic support is added to the solution containing polynucleotides to adsorb them to the support surface before addition of the probe. In this case, hybridization takes place on the support surface. In either embodiment, the relatively long polynucleotides bind to the support through interaction between the cationic surface of the support with the negatively charged polynucleotide backbone. Probe molecules not

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bound to polynucleotide are not bound to the support and remain in solution.

5 The support, and any nucleotide probe bound as a hybrid with the polynucleotide, can be separated from solution by decantation, centrifugation or filtration. If the particles are magnetic, magnetic field séparation can be used. In purification procedures, the nucleotide multimer can be recovered from the solid support by use of elution techniques. In the case of probe assays, the presence of label in the solid phase or in solution can be determined and related to the presence and amount of target nucleic acid sequence contained within polynucleotides in the sample for qualitative and quantitative determinations of diagnostic significance.

10 In all embodiments of the invention, there is provided a highly selective method for separating nucleotide multimers from non-nucleotidic material and for separating mixtures of nucleotide multimers based on their relative lengths. Accordingly, a variety of purification and assay procedures, including qualitative and quantitative procedures, can be carried out according to the invention.

5. Detailed Description of the Invention

25 The well-known technique of nucleic acid hybridization exploits the ability of single stranded nucleotide multimers to combine (hybridize) with complementary strands under appropriate conditions. This invention provides a method for detecting the presence of a polynucleotide which has formed a hybrid with a nucleotide probe. The invention also provides a method for purifying nucleotide multimers and hybrids thereof.

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Any method for the purification of nucleotide multimers or hybridization of polynucleotides in clinical samples must overcome the difficulties posed by biological contaminants which may interfere with separation as well as promote degradation of polynucleotides. In the case of ribosomal RNA ("rRNA"), for example, purification requires the removal of various proteins from the rRNA with which the rRNA is normally complexed. In addition, various nucleases and other enzymes must be inactivated to prevent degradation of the rRNA.

Hybridization normally involves a long target polynucleotide molecule, i.e., a single stranded DNA or RNA consisting of about 100 or more nucleotides (bases). To determine the presence or absence of a particular base sequence (target sequence) within the targeted DNA or RNA, nucleotide probe molecules are synthesized chemically or isolated from biological DNA or RNA through a variety of methods known in the art. The nucleotide probes are complementary to the desired base sequence of the target and are normally labeled with a detectable chemical species. However, the labeling of the nucleotide probe is not absolutely necessary if the hybrid can be detected by other means. Typically, probes are oligonucleotides of from 15-50 bases long, though they may be up to several hundred bases in length. When the target base sequence is present, the nucleotide probe binds to the target through base pairing interactions. Once hybridization is complete, the hybrid is usually separated from the solution which contains unhybridized nucleotide probe molecules. However, in some applications it may be sufficient to merely segregate the soluble and insoluble

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phases, for example, by centrifugation or other techniques which concentrate immobilized hybrid without removing the support from the medium from which the polynucleotide hybrid has been adsorbed. Once the hybrid is segregated or separated free of unhybridized probe, the hybrid is detected by standard methods. Direct labeling methods include radioisotopes, enzymes, fluorescent and chemiluminescent molecules. Indirect labels are also known wherein a chemical species, which is not detectable in and of itself, can be detected when it binds to another complex. An example of indirect labeling is the detection of biotin-labels employing conjugates between streptavidin and enzymes.

Other means of detection which do not require a labeled nucleotide probe could employ intercalators unique to the hybrid, antibodies specific for the hybrid, sensitive gravimetric means, changes in the reflectance of electromagnetic energy, or methods which would permit electronic detection. Of course, in certain cases, it may be desirable to measure unhybridized probe and such procedures are also within the scope of this invention.

When cationic solid supports and appropriate separation solutions are used according to the method of this invention, polynucleotide target molecules bind to the support but unbound nucleotide probes do not bind the support significantly. The precise theoretical explanation of this phenomenon is not entirely clear and the inventors do not wish to be bound to any particular theory, but it is undoubtedly related to charge-charge interactions and the density of cations on the surface of the support. One possible mechanism is that under moderate buffer conditions multiple charged regions of a long polynucleotide cooperatively help bind each other

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to the surface of the cationic solid support. In contrast, shorter nucleotide probes do not possess the same multiplicity of charge-charge binding regions and may thus be less strongly bound to the support.

5 Regardless, the ability of these supports to selectively bind long polynucleotides over oligonucleotides or polynucleotides is clear. This is shown in the examples which follow and points out the particular suitability of the method of this invention for the purification and separation of various polynucleotide mixtures. Unlike

10 the prior art techniques, the method of the invention permits rapid separation of nucleotide multimers on the basis of their length and charge, most notably in hybridization procedures.

15 It is also within the scope of this invention to use as probes analogues of conventional DNA or RNA which have a lower negative charge in the phosphate backbone. Among such analogues may be mentioned the methyl phosphonates described in Miller *et al.*, Biochemistry, 25, 5092 [1986].

20 Some suggestions have been made regarding the use of a nucleotide probe covalently bound to magnetic microspheres to select for a complementary nucleotide strand in solution. But it is known in the art that the covalent bonding of nucleotide multimers directly to solid supports may create steric conditions which are unfavorable for the proper interaction between complementary strands necessary for hybridization. (See, Life Technologies, "Immobilization of Nucleic Acids" WO 25 85/ 04674 [10/24/85]). In the absence of a detailed disclosure in this regard, the method described by Whitehead is inappropriate for purification and hybridization. The present invention, circumvents the need for covalent bonding by relying on ionic

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interactions to effect the separation. Therefore, in a particularly preferred embodiment of the invention, magnetic microspheres bearing a polycationic surface are used to remove nucleotide multimers and hybrids thereof, from solution in any of the various procedures described above.

The methods of the current invention employ two main elements. These are the cationic solid supports and one or more of several contacting solutions employed to facilitate immobilization, separation, washing, and elution steps related to procedures for purifying nucleotide multimers and for assaying for target sequences in polynucleotides based on formation of hybrids with nucleotide probes. These solutions are used for immobilizing polynucleotides, separating polynucleotide hybrids and nucleotide probes, washing immobilized polynucleotides (including hybrids between polynucleotides and nucleotide probes), eluting immobilized polynucleotides, (including hybrids between polynucleotides and nucleotide probes), or eluting a detectable element which correlates with the presence of the immobilized hybrid between polynucleotides and nucleotide probes.

An interplay exists between the composition of the cationic solid support and the formulation of the contacting solution such that the composition of the contacting solution is determined in part by the composition of the cationic solid support and vice versa.

Furthermore, there is a close interplay between many of the contacting solutions since the components of one such solution may be carried over and modified by adding reagents in order to generate the composition needed in the next step of the method.

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In order to clarify the uses of these formulations they can, in general, be broken down into three areas of use. These are:

- 5 I. Purification of polynucleotides or nucleotide multimers.
- 10 II. Immobilization of polynucleotide followed by hybridization typically to test for unique nucleotide sequences contained within said polynucleotides.
- 15 III. The separation from, and detection of, hybrids between polynucleotides and nucleotide probes preformed in solution.

Depending upon which of these procedures one wishes to carry out, a different set of contacting solutions may be required. In general, the steps and solutions useful for each of these procedures, although one or more steps and the use of one or more solutions may not be necessary in every case, is as follows:

- I. Purification of Nucleotide Multimers
 - 20 (1) Immobilization of the nucleotide multimer, usually a polynucleotide, on the cationic solid support in the presence of a SEPARATION SOLUTION.
 - (2) Removal of contaminants employing a WASH SOLUTION and separation of the immobilized multimer on the cationic solid support and the solution phase.
 - (3) Recovery of the multimer from the surface of the cationic solid support using an ELUTION SOLUTION.
- 25 II. Immobilization of Polynucleotides Followed By Hybridization

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- (1) Immobilization of the polynucleotide on the cationic solid support in the presence of a SEPARATION SOLUTION.
- 5 (2) Removal of contaminants employing a WASH SOLUTION (optional).
- (3) Contacting the immobilized polynucleotide with a nucleotide probe in a HYBRIDIZATION SOLUTION designed to minimize immobilization of unhybridized nucleotide probe.
- 10 (4) Removal of unhybridized probe using a WASH SOLUTION which minimizes immobilization of unhybridized nucleotide probe and separation of the immobilized hybrid on the cationic support and in the solution phase (optional).
- 15 (5) Recovery of the hybrid or a detectable element which correlates with the presence of the immobilized hybrid using an ELUTION SOLUTION (optional).
- (6) Detecting the hybrid or a detectable element associated with the hybrid which correlates with the presence of the hybrid, or the unhybridized nucleotide probe.
- 20

III. Separation and Detection of Hybrids Formed in Solution

- 25 (1) Hybridization in the presence of a HYBRIDIZATION SOLUTION between a nucleotide probe and a polynucleotide which contains a base sequence complementary to the nucleotide probe.
- 30 (2) Contacting the hybridization mixture with a cationic solid support and a SEPARATION SOLUTION so as to permit any hybrids formed to bind the cationic solid support without

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permitting significant immobilization of the unhybridized nucleotide probe.

- 5 (3) Removal of unhybridized probe using a WASH SOLUTION which minimizes immobilization of unhybridized nucleotide probe (optional).
- 10 (4) Recovery of the hybrid or a detectable element which correlates with the presence of the immobilized hybrid using an ELUTION SOLUTION (optional).
- 15 (5) Detecting the hybrid or a detectable element which correlates with the presence of the hybrid or the unhybridized nucleotide probe in step (3).

20 As those skilled in the art will recognize, this polycationic support can be used in a variety of assay formats, including direct binding, competition and probe displacement assays. In addition, the labels may be either direct or indirect labels. In some probe displacement assay formats, hybridization could cause label to appear in the unbound, as opposed to the bound phase. The examples above serve solely to illustrate one characteristic of the cationic support to discriminate nucleotid multimers. This invention is not to be limited to any particular assay format.

25 For clarity the further description of this invention will be broken down into the elements relating to the composition of cationic solid support and the formulations of various contacting solutions. However, those skilled in the art will appreciate that actual practice of the invention will be preceded by steps such as sample collection and sample treatment to liberate the nucleotide multimers being purified or probed. For example, the necessity to perform cell lysis and procedures for doing so are well known to the art and a.

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description of such procedures are unnecessary to a complete description of the invention.

CATIONIC SOLID SUPPORTS

The polycationic support matrix of this invention can be selected from a wide variety of inorganic and organic materials including but not limited to metal oxides, glasses, polyamides, polyesters, polyolefins, polysaccharides, polyglycols, and polyaminoacids. The principle requirement is that the matrix support does not unduly adsorb either contaminants or nucleotide probes under the conditions being employed.

Preferred supports are those with a high surface to volume ratio in order to minimize the amount of material needed. Such ratios can be achieved by using particles, particularly micron sized particles, highly porous materials, such as agarose, or small diameter fibers arranged either singly or into a filter mat. The use of small, micron sized particles are preferred.

In the examples which follow, it has been shown that the matrix can comprise a magnetically responsive iron oxide as described by Whitehead, *et al.* (U.S.P.N. 4,554,088; Nov. 19, 1985), a latex microsphere, a sepharose bead, or a suitably functionalized membrane, though the invention is not limited to these materials.

The support should have a cationically modified surface of sufficient charge density to enable the adsorption of the desired polynucleotide under appropriate conditions of salt, detergent and temperature. The charge density may need to be

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determined empirically but generally is in the range of 0.01-10 micro equivalents per milligram of support.

5 The charges may be introduced by a range of cations including but not limited to alkyl and aryl amines including primary amines, secondary amines, tertiary amines, quaternary amines, as well as guanidines and imines.

10 Examples of such moieties include 3-aminopropyl groups, 2-aminoethyl-3-aminopropyl groups, tris (2-aminoethyl) amine, spermine, spermidine, pentamethyl-2-aminoethyl-3-aminopropyl groups, and polymers with amine functionalities such as polylysine, polyarginine, polyethylenimine, and polyallylamine.

15 Functionalities commonly present on the surface of the supports can be readily modified with reagents containing cationic functionalities. Numerous procedures are known in the art for introducing functional groups onto the surface of almost any matrix. See for example, Porath and Axen (*Methods in Enzymology*, 44, 19-45. [1976]), and Goldman *et al.* (in "Biochemical Aspects of Reactions on Solid Supports", G. R. Stark, ed., Academic Press [1971]).

20 In part the formulation of the cationic support is determined empirically depending upon its use. A typical protocol for formulating the solid support follows, but other similar procedures can also be used. If the cationic solid support is to be used solely for the purification of nucleotide multimers, target multimers are tested for their ability to bind to the support in buffers which are less than 0.6 M sodium phosphate, pH = 6.8, and for their ability to be eluted in greater than 20 mM sodium phosphate, pH = 6.8. If the polynucleotide does not bind in less than 0.6 M sodium phosphate, pH = 6.8 the cation density is

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increased or the multiplicity of cations at single attachment sites is increased. (For example, replacing a 3-aminopropyl group with a tris (2-aminoethyl) amine.) On the other hand if the polynucleotide can not be eluted in buffers greater than 20 mM sodium phosphate, pH = 6.8, the cation density is reduced or the multiplicity of cations at single attachment sites is reduced.

When the support is to be used for separating polynucleotide hybrids from nucleotide probes, there is the additional requirement that the nucleotide probe not be significantly bound under conditions which retain the hybrid. These conditions can be tested by monitoring the binding of both the polynucleotide and the nucleotide probe between 20 mM and 0.6 M sodium phosphate, pH = 6.8, and selecting a buffer concentration which gives a maximum binding ratio of polynucleotide to nucleotide probe. If satisfactory binding ratios cannot be achieved, the surface density and multiplicity of cations is further modified.

It should be noted that generally the length of the polynucleotide to the nucleotide probe should usually be in the ratio of greater than 3 to 1 for good separation, and is preferably greater than 5 to 1. However, the use of DNA or RNA analogues having lower negative charge in the probe backbone can permit relaxation of this criterion. In the case of some probe displacement and competition assays, however, the 3 to 1 ratio need not be met and, in fact probe constructions may be longer than the target.

CONTACTING SOLUTIONS

In addition to the polycationic support, the separation solution is of particular importance and

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should be carefully designed to facilitate immobilization and to prevent degradation of the nucleotide multimers and their probe hybrids. The contacting solutions can include various buffers, the concentrations of which will vary depending upon the operation desired. The components of the solution and their concentrations of the solution are dependent upon a number of factors: the need, if any, to inactivate nucleases, especially in clinical samples; the polynucleotide separation desired; and other steps desired after separation. If, for example, a subsequent enzyme detection scheme is to be included, protein denaturants or inhibitors may be necessary as part of the separation solution in order to maximally reduce background from endogenous enzyme activity.

The components which remain from previous procedural steps must also be considered. For example, in most cases separations will be done by removing polynucleotides from biological material or hybridization reactions. Various components and reagents which are used either to liberate said polynucleotides from biological samples or are used to facilitate hybridization (i.e., accelerated rate hybridization) will remain as components of the subsequent separation solution.

The contacting solutions have many components in common and in some cases their compositions may be identical. For example, the composition of the wash solution and the separation solutions may be similar in regard to inhibiting nuclease activity, preventing the binding of nucleotide probes, and in negating adverse affects of substances associated with hybridization rate acceleration.

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The first step in formulating the contacting solutions is dependent upon the desired use of the cationic solid support. The next step is to formulate the contacting solutions. In general the reagents for formulation are selected from the following categories.

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ENZYMES - These are used in part to degrade and inhibit nucleases, disrupt protein/nucleic acid interactions, and in some cases cleave label which is associated with immobilized hybrids. They may include proteases, diesterases, nucleases and peptidases.

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NUCLEASE INHIBITORS - These agents are employed to prevent degradation of both polynucleotide molecules and nucleotide probes. They include ribonuclease inhibitors such as vanadyl sulfate, vanadyl ribosides and RNAGuardTM (human placental ribonuclease inhibitor, Pharmacia Fine Chemicals, Piscataway, NJ, USA), deoxyribonuclease inhibitors, and detergents such as sodium dodecylsulfate and lithium lauryl sulfate which are general protein denaturants.

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CHELATING AGENTS - These substances are utilized to chelate various metals which when unchelated are essential for the activity of various enzymes. For example, many deoxyribonucleases require Ca^{2+} for activity. Chelation of metals ions is also important for optimizing hybridization reactions since metals such as Mn^{2+} can interfere with hybridization. Chelating agents include ethylene diaminetetraacetic acid (EDTA) and ethylene glycol bis. (2-aminoethyl ether)-N, N,N',N' - tetracetic acid (EGTA).

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DETERGENTS - These substances are used to help solubilize particulate material, inhibit nucleases, and reduce unwanted immobilization of contaminants and nucleotide probes to cationic solid support employing Separation Solutions and Wash Solutions. Detergents may also be used to accelerate hybridization reactions.^{2/} In some circumstances one detergent may be used to abolish the adverse effects of a second detergent by forming mixed micelles. Substances in the detergent category include sodium dodecyl sulfate, lithium lauryl sulfate, N-lauroylsarcosine, sodium diisobutyl sulfosuccinate, Triton^R X-100, Brij^R, Tween^R-20 and Tween^R-80.^{3/}

BUFFER SALTS - These substances are employed to maintain pH ranges which are compatible with nucleotide multimers stability, hybrid stability, and the stability of agents used as labels. These substances are also used to establish the primary balance between immobilization of hybrids on the cationic solid support and retention of nucleotide probes in solution using Separation Solutions. In addition, they are employed to establish the stringency in the Hybridization Solutions. The concentration of various salts can also be used to assist the elution of hybrids or a nucleotide probe associated with a hybrid from the surface of the support. Such substances

^{2/} Patent pending on accelerated rate system.

^{3/} Triton^R, Brij^R, and Tween^R are registered trademarks of ICI Americas.

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may include salts of potassium, sodium, calcium, guanidine, chloride, fluoride, sulfate, phosphate, acetate, succinate, phytic acid, and tripolyphosphate.

5 ORGANIC SOLVENTS - These solutions are used to help emulsify various substances, alter hybridization temperatures and conditions, remove contaminating substances and assist in the removal of nucleotide probes and hybrids employing elution solutions. Such organic solvents may include methanol, ethanol, isopropylalcohol, formamide, and dimethylformamide.

10 15 OTHER ORGANIC AND INORGANIC SUBSTANCES - Other substances may be used in various contacting solutions in order to impart a desired property. These include organic and inorganic acids such as acetic acid, phosphoric acid, hydrogen fluoride, sulfuric acid, and nitric acid. They also include inorganic substances which might be used to remove the label from a nucleotide probe employing an elution solution. These may include periodate, lead acetate and manganese dioxide.

20 25 The individual contacting solutions which may be used in specific cases are generally formulated as follows:

30 HYBRIDIZATION SOLUTION - Formulated generally with a salt buffer, detergents, nuclease inhibitors and chelating agents. The formulation is compounded to facilitate hybridization between a polynucleotide and nucleotide probe. Furthermore, when the

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polynucleotide is immobilized prior to hybridization the formulation is selected to preclude significant binding of the nucleotide probe to the cationic support.

5 SEPARATION SOLUTION - Comprises generally a salt buffer, detergents, and nuclease inhibitors so as to permit immobilization of the hybrid without permitting the significant immobilization of the nucleotide probe.

10 WASH SOLUTION - Formulated generally with a salt buffer and detergents so as to keep the hybrid immobilized while permitting removal of contaminants and unhybridized nucleotide probe.

15 ELUTION SOLUTION - Comprises generally a salt buffer, organic solvents, detergents and other reagents so as to liberate a polynucleotide, polynucleotide hybrid, nucleotide probe, or a label associated with a hybrid from the surface of the support.

20 DETECTION SOLUTIONS - Formulated specifically to detect the hybrid or a specific label. Its composition is dependent on the detection means and is formulated according to prior art methods. For example, if the label is an enzyme, the detection solution will contain the selected enzyme substrate and other reagents.

25 Persons skilled in the art and having the benefit of the foregoing general description will be able to tailor the components of specific contacting solutions to a wide variety of procedures and conditions well within the skill of the art, particularly with

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reference to the following examples. Materials used in
the following examples include: Magnetic amine
microspheres were obtained from Advanced Magnetics, Inc.
(Cambridge, MA, USA; Biomag M4100, 50 mg/ml in water);
5 lysozyme was Grade I from Sigma, St. Louis, MO, USA;
urea was enzyme grade from Bethesda Research Labs,
Bethesda, MD, USA; RNAGuard™ (human placental
ribonuclease inhibitor) from Pharmacia, Piscataway, NJ,
USA; Cytoscint™ (liquid scintillation cocktail) from
WestChem, San Diego, CA, USA; polyethylene glycol 8000
10 from Eastman-Kodak, Rochester, NY, USA; diisobutyl
sulfosuccinate (DIBSS), see Examples 7 and 12, used in
Gen-Probe, Inc. accelerated rate system (Patent
Pending), from Mona, IN, USA; hydroxyapatite (HAP) and
15 Zwittergent 3-14 were from Behring-Diagnostics,
Calbiochem Division, La Jolla, CA, USA; sodium dodecyl
sulfate (SDS) was from Sigma, St. Louis, MO, USA, as was
Trizma-base (Tris); Triton X-100 was from Fisher
Diagnostics, Orangeburg, NY, USA; stock [³H]-rRNA (16S
20 and 23S subunits of the E. coli ribosome). The rRNA of
the 16S subunit is approximately 1500 bases long; the
23S subunit is approximately 3000 bases. All other
reagents were reagent grade. All phosphate buffers (PB)
25 were the sodium salt, pH 6.8 unless
otherwise stated. All manipulations were performed at
room temperature in 1.5 ml screw-capped polypropylene
microcentrifuge tubes unless otherwise stated.

Example 1

The Binding of rRNA to Magnetic Amine Microspheres

30 To determine the effects of various buffer
concentrations on rRNA binding, mixtures for separation
were prepared by combining 5 µl of magnetic amine

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microspheres, 1 μ l(1 μ g) of [3 H]-rRNA and 100 or 150 μ l of the reagents listed below in Table 1. The reagents include those which would be used in a complete assay protocol and were studied to determine their affect on rRNA binding. The mixtures were vortexed lightly for 2 seconds and allowed to stand for 5-10 minutes. The magnetic amine microspheres were magnetically pelleted using a BioMag Separator (Advanced Magnetics, Inc. catalog #M4101) to the bottom of the tube and the supernatants (non-bind) were removed. The magnetic amine microspheres were then washed (once or twice) by vortexing them for 1-2 seconds in 100 or 150 μ l of the buffer in which binding had taken place, magnetically pelleting the magnetic amine microspheres and removing the supernatants. The magnetic amine microspheres were then resuspended in 100 or 150 μ l of wash buffer and added to 15 ml of Cytoscint in 20 ml polypropylene tubes as were the non-bind and wash fractions. The amount of tritium in each sample was then determined using a Nuclear Chicago scintillation counter.

As shown in Table 1 below, the removal of rRNA by adsorption to magnetic amine microspheres is sensitive to changes in buffer concentration but is not unduly affected by other reagents that would be present in a complete assay protocol. More importantly, the effects of reagents that do decrease binding can be manipulated by varying their concentrations or by adding other reagents into the solution.

Table 1

<u>Reagent System</u>	<u>% [3H]-rRNA Bound to Microspheres</u>
H ₂ O	94

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	50mM PB	99
	100mM PB	98
	140mM PB	92
	200mM PB	89
5	300mM PB	14
	1M NaCl	99
	1M NaCl + 50mM PB	95
	Tris, PEG ^{4/}	99
10	Sucrose, Lysozyme ^{5/}	99
	8M urea	74
	4M urea	97
	1% Triton X-100	100
	5% Triton X-100	47
	1% Zwittergent 3-14	98
15	5% Zwittergent 3-14	78
	2M urea + 1M LiCl	73
	10mM EDTA in 50mM PB	86
	500 U/ml RNAGuard TM	100
	67% HOAc	37
20		

Table 1 (cont'd)

<u>Reagent System</u>	<u>%[³H]-rRNA Bound to Microspheres</u>
50% HOAc	75
33% HOAc	79
20% HOAc	99

^{4/} 0.1M Tris (pH 7.5), 1M NaCl, 2.5% polyethyleneglycol.

^{5/} 7% sucrose, 0.08M glycine (pH 9.0 w/NaOH), 8mM EDTA, 25mM DTT, 2 mg/ml lysozyme.

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	pH 4 NaOAc ^{6/}	95
	18% DIBSS ^{7/}	25
	18% DIBSS, 50mM PB	35
	18% DIBSS,	
5	5% Triton X-100, 50mM PB	90
	18% DIBSS, 1% SDS,	
	5% Triton X-100, 50mM PB	80

Example 2
The Binding of DNA to Magnetic Amine Microspheres

10 This study was designed to determine the effects of different buffer concentrations and other assay reagents on DNA binding to magnetic amine microspheres. The method of Example 1 was used, except [³H]-cDNA against rRNA from Mycoplasma pneumonia was used instead of stuct rRNA. The cDNA was a mixture of cDNAs ranging in size from approximately 400 to 1600 bases.

15 As shown in Table 2, cDNA binds to magnetic amine microspheres about as well as rRNA and the extent of binding can be manipulated by variations in buffer concentrations as well as the addition of other reagents.

Table 2

25	<u>Reagent System</u>	<u>% [³H]-rRNA Bound to Microspheres</u>
	H ₂ O	100

30 ^{6/} HOAc adjusted to pH 4 w/5N NaOH. Final HOAc conc=44%.

7/ wt: vol diisobutyl sulfosuccinate.

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	0.05M PB	100
	0.10M PB	99
	0.15M PB	99
	18% DIBSS, 5% Triton	
5	X-100, 100 mM PB	0
	14.5% DIBSS, 3.75%	
	Triton X-100, 7.5 mM PB	0
	9% DIBSS, 2.5% Triton	
	X-100, 50 mM PB	89
10	4% DIBSS, 5% Triton	
	X-100, 50mM PB	100

Example 3

The Elution and Hybridization of rRNA from Magnetic Amine Microspheres

15 In this experiment, [³H]-rRNA was eluted from magnetic amine microspheres and hybridized. In addition to the materials described in Example 1, [³²P]-ATP was obtained from Amersham Corp., Arlington Hts., IL, USA, and T4 polynucleotide kinase from Bethesda Research Labs, Inc., Gaithersburg, MD, USA.

20 A probe was synthesized using an Applied Biosystems, Inc. Model 380A DNA Synthesizer. A deoxyoligonucleotide was produced with the sequence "5'-AGGACCGTTATAGTTAGGGCCGCCGT-3'" using standard phosphoramidite chemistry (this sequence is complementary to the bases 1901-1926 of the 23S subunit of the E. coli ribosome) (Patent pending on probe sequence.) The oligomer was then labeled on the 5' end using [³²P]-ATP and T4 polynucleotide kinase according to the procedure of Maxam and Gilbert (Proc. Natl. Acad. Sci. U.S.A., 74, 560 (1977)).

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[³H]-rRNA immobilization, elution and hybridization: [³H]-rRNA was immobilized by combining 10 μ l of magnetic amine microspheres (50 mg/ml), 100 μ l of 0.14M PB and 3 μ l of [³H]-rRNA (1 mg/ml). The mixture was lightly vortexed 1-2 seconds and allowed to stand 5-10 minutes. The magnetic amine microspheres were magnetically pelleted and the supernatant (non-bind) was removed. The magnetic amine microspheres were then washed one time with 100 μ l of 0.14M PB and the supernatant removed.

In order to elute the [³H]-rRNA, 50 μ l of 0.6 M PB were added to the magnetic amine microspheres and the mixture was vortexed 1-2 seconds. Five microliters were removed and counted for radioactivity (See Example 1). The remainder of the solution was allowed to stand for 15-30 minutes at room temperature. The magnetic amine microspheres were then magnetically pelleted and a 5 μ l aliquot of the supernatant was counted for radioactivity. Another aliquot of the supernatant was removed and placed in the following hybridization mixture: 25 μ l eluted [³H]-rRNA in 0.6 M PB (1.1 pmol), 0.5 μ l [³²P]-DNA probe (.23 pmol), 1.5 μ l 1% SDS, and 4 μ l H₂O totaling 31 μ l of solution.

A control hybridization was also performed using the same amount (based on radioactivity) of stock [³H]-rRNA: 1.8 μ l stock [³H]-rRNA (1.1 pmol), 0.5 μ l [³²P]-DNA probe (.23 pmol), 14.8 μ l 1.0M PB, 1.5 μ l 1% SDS, and 12.4 μ l H₂O totaling 31 μ l of solution.

The hybridization mixtures were then incubated for 3 hours at 50°C. Each mixture was added to 5 ml of 0.14M PB containing 2% hydroxyapatite (HAP) in a 7 ml polypropylene scintillation vial. After vortexing for 15 seconds the samples were incubated for 5 minutes at 50°C. The HAP was then pelleted by centrifugation for 2

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5 minutes in an IEC table top clinical centrifuge (Needham Hts., MA, USA) at 2000 X G. The supernatant was removed, 5 ml of 0.14 M PB added and the mixture vortexed 15 seconds, followed by centrifugation as described above. The supernatant was removed and the HAP, non-bind and wash solutions were counted for Cerenkov [^{32}P] activity in order to determine percent hybridization, i.e., the percent of the probe associated [^{32}P] counts bound to the HAP.

Example 4

The Binding of rRNA in Urine to Magnetic Amine Microspheres

This experiment was undertaken to determine the effect of urine on rRNA binding to magnetic amine microspheres. In addition to the materials described in Example 3, fresh urine samples were obtained from volunteers.

To 100 μ l fresh urine was added 200 μ l of 50mM PB, 8M urea or HOAc, pH 4 (glacial acetic acid was adjusted to pH 4 with the addition of 0.28 ml of 10N NaOH per ml of HOAc) as well as 1 μ l stock [3 H]-rRNA (1 μ g) and 5 μ l magnetic amine microspheres. The mixtures were worked up as described in Example 1, except that each was washed with 200 μ l of 50mM PB.

The results showed that in the presence of urine, the urea, which is used with biological samples

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to free nucleic acids and denature proteins and nucleases, decreases the binding of [³H]-rRNA to 0.8%. In urine plus phosphate buffer alone, only 13% of the [³H]-rRNA bound to the support. Only in combination with HOAc, pH 4 did 100% of the rRNA bind to the magnetic amine microspheres.

Example 5

The Binding of rRNA Suspended in Sputum to Magnetic Amine Microspheres

This study was undertaken to determine the proper combinations and concentrations of buffers and reagents necessary to bind [³H]-rRNA in more complex biological samples. The materials described in Example 4 plus pooled, frozen sputa (each pool containing sputum from several patients) were obtained from various hospitals.

Sputum samples were first liquified (immediately before use) by adding 1/10 volume of 0.5M dithiothreitol (DTT), followed by vortexing and incubation for 10-15 minutes at 22°C. A variety of reagents were then added to 100 µl aliquots of the liquified sputum, along with 1 microliter stock [³H]-rRNA (1 µg) and 5 µl magnetic amine microspheres. After 5 minutes of incubation at 22°C, the samples were then processed as in Example 1 (with the exception that each was washed with 200 µl 50 mM PB) to determine the extent of [³H]-rRNA binding. The results are summarized in Table 3.

Table 3

Reagent Systems for [³H]-rRNA removal from Sputum with Magnetic Amine Microspheres

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	<u>Sample Composition</u>	<u>Final Concentrations</u>	% [³ H]-rRNA Bound to	Magnetic Amine
		<u>HOAc^{8/}</u>	<u>Urea</u>	<u>Microspheres</u>
5	100 µl sputum + 100 µl HOAc + 300 µl 50mM PB	20%	--	43
	100 µl sputum + 100 µl HOAc	50%	--	8
10	100 µl sputum + 256 µl pH 4 HOAC	56%	--	41
	100 µl sputum + 256 µl pH 4 HOAC + 150 µl 8M urea	39%	2.4M	50
	100 µl sputum + 300 µl HOAc/urea ^{9/}	50%	4.0M	81
15				

Example 6
The Hybridization of rRNA Purified from Sputum

20 In this experiment, the [³H]-rRNA removed from sputum and bound to the support was eluted and studied to determine whether the separation method used in the invention unduly decreases hybridizability.

A deoxynucleotide probe with the sequence "5'-GGCCGTTACCCCACCTACTAGCTAAT-3'" (complementary to bases 235-260 of the 16S E. coli ribosome)^{10/}, was

25 ^{8/} That is, partially neutralized HOAc.

^{9/} HOAc/urea, pH 5, was made by combining 840 ul pH 4 HOAc (see Example 4) with 320 mg urea (final volume = 1ml, [urea] = 5.33M, 66% partially neutralized HOAc).

^{10/} Patent pending on probe sequence.

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synthesized and labeled with [³²P] as described in Example 2.

After binding of [³H]-rRNA in sputum to magnetic amine microspheres and removal of the non-bind fraction (see Example 5), the magnetic amine microspheres were washed one time with 1M NaCl, 50 mM PB. After removal of the supernatant, 50 μ l of 0.6M PB were added and the mixture incubated 30 minutes at 22°C or for 15 minutes at 72°C with occasional mixing. The magnetic amine microspheres were then magnetically pelleted and the percent [³H]-rRNA eluted was determined by comparison of the radioactivity of aliquot of the supernatant with an aliquot of the magnetic amine microspheres before incubation (see below).

The ability of the eluted [³H]-rRNA to hybridize to a complementary DNA-probe (see Materials) was determined by comparing hybridizability of eluted rRNA with stock rRNA, both in target excess and probe excess. The following hybridization mixtures were prepared:

- (1) 30 μ l eluted rRNA in 0.6M PB (.44 pmol), 1 μ l DNA-probe (.07 pmol), 4 μ l 1% SDS, and 2.5 μ l H₂O, totaling 37.5 μ l.
- (2) 0.7 μ l stock rRNA (.44 pmol), 29.3 μ l 0.6M PB, 1 μ l DNA-probe (.07 pmol), 4 μ l 1% SDS, and 1.6 μ l H₂O totaling 36.6 μ l.
- (3) 9.35 μ l eluted rRNA in 0.6M PB (.14 pmol), 1 μ l DNA-probe (.7 pmol), and 1.34 μ l 1% SDS totaling 11.69 μ l.
- (4) 0.22 μ l stock rRNA (.14 pmol), 9.13 μ l 0.6M PB, 1 μ l DNA-probe (.7 pmol) and 1.06 μ l 1% SDS totaling 11.41 μ l.

The hybridization mixtures were then incubated for 2 hours at 50°C, followed by isolation of hybrids on

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HAP as described in Example 2. In target excess, eluted rRNA showed 67% hybridizability as compared to stock rRNA. In probe excess, eluted rRNA showed 65% hybridizability compared to stock rRNA.

5

Example 7

The Recovery of RNA/DNA Hybrids from Buffer Solutions
Using Magnetic Amine Microspheres

10

This experiment was designed to study magnetic amine microspheres as a separation support for RNA/DNA hybrids formed in solution. The materials were the same as those described in Example 3. In addition, a Legionella specific DNA probe (average length 100 bases) was used.

15

The ability of magnetic amine microspheres to separate RNA/DNA hybrids was studied in two different systems.

20

System 1: An RNA/DNA hybrid was formed by combining 8 μ l stock [3 H]-rRNA (5 pmol), 1 μ l deoxyoligonucleotide probe (.5 pmol), 10 μ l 0.28 M PB and 1 μ l 1% SDS, and incubating this mixture for 1.5 hours at 50°C. Additionally, a control mixture was made exactly as the hybrid mixture, except that the 8 μ l of target RNA was replaced with 8 μ l of water. This control mixture was also incubated for 1.5 hours at 50°C. Half of each mixture (10 μ l) was added to 5 ml of 2% HAP in 0.14M PB and worked up as described in Example 2. The other half of each mixture was added to 200 μ l 0.14M PB + 10 μ l magnetic amine microspheres and worked up as described in Example 1 (using 0.14M PB for washing).

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System 2: An RNA/DNA hybrid mixture was made by combining 4 μ l Legionella rRNA (2.5 fmol), 2 μ l Legionella probe (1.3 fmol) and 196 μ l containing 4% DIBSS, 30mM PB and 3% SDS. A control mixture was made exactly as the hybrid mixture, except that the 4 μ l of target RNA was replaced with 4 μ l of water. Both mixtures were incubated for 2 hours at 72°C. A 50 μ l aliquot of each mixture was added to 5 ml of 2% HAP in 0.14M PB and worked up as described in Example 2, except at 72°C and two washes. Another 50 μ l of each mixture was added to a separation mixture such that the final conditions were 150 μ l total volume, containing 18% DIBSS, 5% Triton X-100, 0.1M PB and 10 μ l magnetic amine microspheres. The magnetic amine microspheres were then worked up as described in Example 1, except at 72°C and two washes (wash 1 was 18% DIBSS, 5% Triton and 0.1M PB; wash 2 was 5% Triton, 0.1M PB). The results are shown in Table 4.

Table 4

	Method of Separation	% Bound			
		System 1		System 2	
		<u>Hybrid</u>	<u>Control</u>	<u>Hybrid</u>	<u>Control</u>
	HAP	53	1.5	30.3	0.4
	Magnetic Amine Microspheres	51	0.5	28.6	0.2

This result indicates that the magnetic amine microspheres are capable of recovering RNA/DNA hybrids from buffer solutions, while leaving unhybridized probe in solution.

30

Example 8

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The Recovery of DNA/DNA Hybrids from Solution Using
Magnetic Amine Microspheres

This experiment was undertaken to study magnetic microspheres as a separation support for DNA/DNA hybrids formed in solution. In addition to the materials described in Example 2, a synthetic 36-base deoxyoligonucleotide probe complementary to a 36-base region of the cDNA described in Example 2 was used.

The oligonucleotide was labeled with [³²P] as described in Example 3. A DNA/DNA hybrid mixture was made by combining 20 μ lcDNA target (approximately 20 fmol), 1 μ lDNA probe (approximately 60 fmol) and 200 μ lcontaining 44% DIBSS, 30mM PB and 3% SDS. A control mixture was made exactly as the hybrid mixture, except that the 20 μ l of target was replaced with 20 μ l of water. Both mixtures were incubated for 2 hours at 60°C. A 50 μ l aliquot of each mixture was added to either 450 μ l of 2% HAP in 0.08M PB or 450 μ l of 5% Triton, 45 mM PB plus 10 μ l of magnetic amine microspheres. Each separation mixture was incubated at room temperature for 5 minutes, the HAP or the microspheres were pelleted as described in previous examples and the supernatants were removed. The pellets were washed one time at room temperature with 500 μ l of either 0.08 M PB (HAP) or 5% Triton, 45 mM PB (microspheres) as described in previous examples. All fractions were then dissolved in 15 ml Cytoscint and counted for radioactivity as described previously. The results in Table 5 compare favorably with those for System 2 in Example 7 indicating that binding of hybrids is charge dependent and unaffected by whether the target is RNA or DNA.

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Table 5

	<u>Method of Separation</u>	% Bound <u>Hvbrid</u>	% Bound <u>Control</u>	Net % <u>Hybridization</u>
5	HAP	33.9	1.1	32.8
	Magnetic Amine			
	Microspheres	36.1	4.4	31.7

Example 9

Study of Elution Buffers for Removal of Labeled
Nuclceotide Probes Associated with Nucleic
Acid Hybrids Bound to Magnetic Amine Microspheres

The potential of various elution buffers for removal of labeled nucleotide probes associated with DNA/RNA hybrids bound to magnetic amine microspheres was demonstrated by the following methods.

Method 1: A [¹²⁵I]-labeled deoxyoligonucleotide probe (prepared by standard methods) was hybridized to target RNA in 0.5 M PB, pH 5 (30 minutes at 60°C, 30 µl total volume). Then, 1 ml of 0.25M PB, pH 5, 0.05% Triton X-100 and 2.5 mg magnetic amine microspheres was added, vortexed, and incubated 5 minutes at 60°C. The spheres were washed 2 times with 0.25M PB, pH 5, 0.05% Triton X-100. Potential elution agents were tested by adding 100 µl to aliquots (5%) of the magnetic amine microspheres, incubating 5 minutes at 60°C, separating supernatant from spheres and measuring the amount of [¹²⁵I] present in each fraction using a gamma counter (Iso-Data, Palatine, IL, USA, Model 20/20DM). Results are summarized in Table 6.

30 Elution Agent

Table 6

Percent Eluted

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	0.25M PB, pH 5, 0.01% SDS, 50% formamide	95
	3M NaOAc, pH 5	95
	0.25M PB, pH 5, 0.01% SDS, 50% DMSO	85
	0.1M NaOAc, pH 5, 1% SDS, 1M Guanidinium-HCl	70
5	0.1M NaOAc, pH 5, 1% SDS, 1M sodium propionate	68
	0.1M NaOAc, pH 5, 0.01% SDS, 0.1M sodium pyrophosphate	65
	1M sodium citrate, pH 5	60
	0.25M PB, pH 5, 5% SDS	56
10	0.1M NaOAc, pH 5, 1% SDS, 0.1M MgCl ₂	40

These results demonstrate that a wide range of reagents are capable of removing labeled nucleotide probes from RNA/DNA hybrids bound to microspheres.

15 Method 2: To demonstrate that other elution reagents are capable of removing labeled nucleotide probes associated with DNA/RNA hybrids bound to amine magnetic microspheres, the following protocol was used to test these potential elution reagents.

20 A [³²P]-labeled deoxyoligonucleotide probe (prepared as described, supra) was hybridized to target rRNA in 0.48 M PB (pH 5)/0.1% SDS (total volume 150 µl) for 30 minutes at 60°C. Then, 1 ml of 0.15 M PB (pH 6.8)/5.0% Triton X-100 and 2.5 mg of amine magnetic microspheres was added, vortexed and incubated 5 minutes at 60°C. The microspheres were washed three times with 0.3 M PB (pH 6.8). Elution reagents were then tested by adding 300 µl of the elution solution to the amine magnetic microspheres, incubating 5 minutes at 60°C, separating supernatant from spheres and measuring the amount of [³²P] present in each fraction using a scintillation counter (Delta 300 Scintillation System, Searle Analytical, Inc., Des Plaines, IL, USA). Results are summarized in Table 7.

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Table 7

	<u>Elution Agent</u>	<u>Percent Eluted</u>
5	0.05 M Phytic Acid, pH 8.0	91%
	0.05 M Tripolyphosphate, pH 8.0	87%
	0.3 M PB (pH 6.8)/50% formamide	86%

These results demonstrate that polyphosphates can efficiently elute nucleic acids from magnetic amine microspheres.

10

Example 10
Use of Magnetic Amine Microspheres as a Solid
Phase Hybridization Support

15

In this experiment, target rRNA was bound to magnetic amine microspheres and then hybridized. To 5 μ l of magnetic amine microspheres were added 100 μ l 0.14M PB and 2 μ l stock [3 H]-rRNA (2 μ g, 1.26 pmol). The materials were the same as those described in Example 3. The mixture was incubated 10 minutes at 22°C, the magnetic amine microspheres magnetically pelleted and the supernatant removed. To the magnetic amine microspheres were added 20 μ l 0.14M PB and 0.5 μ l probe (.23 pmol). As a control, hybrids were formed in a solution of 2 μ l stock [3 H]-rRNA, 0.5 μ l probe, 5 μ l 0.28M PB, 0.5 μ l 1% SDS and 2 μ l H₂O. Both hybridization mixes were then incubated 3 hours at 40°C. The magnetic amine microspheres were washed 3 times with 100 μ l 0.14M PB as described in Example 1. The results show the extent of hybridization of probe was 5% in the case of immobilized rRNA as compared with 20% for hybridization in solution (control).

20

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Example 11

Use of Magnetic Amine Microspheres to Purify Nucleic
Acids from Cell Lysate

This experiment was undertaken to show that
5 the magnetic amine microspheres described, supra, can be used to purify RNA from a crude sample without significantly reducing hybridizability. The method of purifying rRNA was studied using rRNA from Legionella pneumophila. The materials were the same as those described in Example 5. In addition, a Legionella specific probe and Legionella pneumophila organisms were obtained. San Diego, CA, USA.

Cell lysis and release of rRNA was achieved by combining 30 μ l water, 5 μ l Legionella pneumophila suspension (5×10^5 organisms) and 5 μ l 24% SDS, .08M Tris-base, .08M EGTA and .08M EDTA, followed by incubation for 30 minutes at 72°C. One quarter of the sample (10 μ l) was then assayed directly for rRNA using the following assay mixture: 10 μ l sample + 114 μ l 5.94M PB + 1 μ l DNA-probe¹¹ + 75 μ l water. The mixture was incubated 30 minutes at 72°C and was then analyzed for hybrid formation using HAP as described in Example 2, except the HAP binding step was done at 72°C instead of 50°C. To another 10 μ l of sample was added 30 μ l HOAc/urea (see Example 5), the mixture was vortexed 5 seconds, 5 μ l of magnetic amine microspheres were added and the mixture was lightly vortexed (about 3 seconds). After incubating 5 minutes at 22°C and subsequent washing with 150 μ l 1M NaCl, 50mM PB at 22°C, the bound

30 11/ Patent pending on accelerated rate system.

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rRNA was eluted with 50 μ l 0.6M PB (details described in Example 2). The eluant was then assayed for rRNA by combining 50 μ l sample, 109 μ l 5.94M PB, 1 μ l DNA-probe and 40 μ l water, incubating the mixture for 30 minutes at 72°C, and analyzing hybrid formation using HAP (see Example 2). Results are summarized in Table 8.

Table 8

	<u>% Hybridization</u>
10	Direct Assay
	30
	Microsphere purification, elution, assay
	21

Example 12
Purification of Nucleic Acids from Sputum with
Magnetic Amine Microspheres

15 In this experiment, the use of magnetic amine microspheres for the purification of Legionella pneumophilia rRNA from sputum and subsequent hybridizability of said rRNA was demonstrated. The materials were the same as those in Example 7. A pooled sputum sample was liquified as described in Example 5. To 30 μ l aliquots of this sputum sample or 30 μ l aliquots of water were added 5 μ l of a Legionella pneumophila suspension (2×10^6 , 2×10^5 or 2×10^4 cells per 5 μ l). Using the same procedure as described in Example 11, the cells were lysed, one half of each sample was hybridized directly (20 μ l sample and 65 μ l water used here) and one half was hybridized after purification of the rRNA using magnetic amine microspheres (20 μ l sample and 60 μ l HOAc/urea used

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here). Hybrid formation was analyzed using HAP (see Example 2). The results are shown in Table 9.

Table 9

		<u>% Hybridization</u>
5	Water	10^6 organisms 42
		10^5 organisms 33
		10^4 organisms 5.9
10	Sputum	10^6 organisms 67
		10^5 organisms 13
	Background	10^4 organisms 2.6 (no organisms) 1.2

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Example 13

Accelerated Solution Hybridization^{12/} of rRNA from Legionella pneumophila with Separation of the Hybrid on Magnetic Amine Microspheres

In this experiment, the use of magnetic amine microspheres for the detection of Legionella pneumophila rRNA in sputum was demonstrated with accelerated solution hybridization. The materials were the same as those described in Example 11, except that individual sputa were used instead of pooled sputa. Additionally, 5 ml screw-capped tubes were purchased from Vanguard (Sweden); glass beads (0.2 - 0.3 mm) from Glen Mills, Inc., Maywood, NJ, USA; the ultrasonic cleaner from Branson Equipment Co., Shelton, CT, USA (Model 1200); and the Corning magnetic separator rack from Advanced Magnetics, Inc. (catalog #M4700).

Individual sputa were liquified as described in Example 5 for pooled sputa. The liquified sputa were then seeded with Legionella pneumophila (see Example 10) such that 0.1 ml contained 10^4 organisms. To 5 ml tubes were added 100 μ l glass beads (acid washed), 100 μ l lysis buffer (20% SDS, 10mM EDTA, 10mM EGTA, 50 mM Tris, pH 8.0) and 0.1 ml seeded sputum or 0.1 ml negative control (1 mg/ml calf thymus DNA in 0.1% SDS), all in triplicate. The samples were then sonicated for 15 minutes at 72°C, 2 ml probe solution (44% DIBSS, 50mM PB, pH 6.8, 10,000 cpm Legionella specific DNA probe per 2 ml) was added, the mixture was vortexed and then incubated for 1 hour at 72°C. The hybrids were then

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^{12/} Patent pending on accelerated rate system.

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isolated according to one of the following procedures (all in triplicate): 1. HAP, centrifugation - 2 ml of separation solution (5% HAP in 0.26 M PB, pH 6.8, .02% SDS) was added to each tube, the tubes were inverted 5X, incubated at 72°C for five minutes, inverted 20X, then centrifuged for 2 minutes at 2000xg. The supernatant was decanted off and discarded, 4.5 ml wash solution (0.14M PB, pH 6.8, .02% SDS) was added, each tube was vortexed 20 seconds, and the HAP was pelleted and the supernatant decanted as described above. Each tube was then counted for radioactivity in a Berthold gammacounter. 2. Magnetic amine microspheres, centrifugation - 2.75 ml magnetic separation solution (9.5% Triton, 0.15M PB, pH 6.8, 150 l magnetic amine microspheres per 2.75 ml solution) was added to each tube, and the tubes were worked up as in procedure 1 with the following exceptions: wash solution was 18% DIBSS, 5% Triton, 0.1M PB; after addition of wash solution, the tubes were inverted to remix the magnetic amine microspheres. 3. Magnetic amine microspheres, magnetic separation - this procedure is exactly like procedure 2 with the exception that centrifugation is replaced with magnetic separation using the Corning magnetic separator rack. The results are shown in Table 10.

Table 10

% cpm bound

<u>Method</u>	<u>Control</u>	<u>Hybrid</u>	<u>Hybrid:Control</u>
1	1.9	8.2	4.3
2	0.75	5.1	6.8
3	0.48	5.5	11.5

Method 1 = HAP, centrifugation. Method 2 = magnetic amine microspheres, centrifugation. Method 3 = magnetic

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amine microspheres, magnetic separation. Control values represent the average of 4 separate determinations, each done in triplicate. Hybrid values represent the average of 30 separate determinations (30 individual sputa) each done in triplicate.

Example 14

Use of Magnetic Propylamine Microspheres
to Separate rRNA

To show that the method of the invention is not limited to the properties of a single type of magnetic cationic microsphere

as a support surface but includes other cations, [³H]-rRNA was removed from defined buffers with magnetic propylamine microspheres. The materials were the same as those described in Example 1, except that the microspheres were N-3-aminopropyl silane magnetic microspheres (Advanced Magnetics, Inc., special order).

The method used here for removing [³H]-rRNA was exactly the same as that used in Example 1, except that magnetic propylamine microspheres were used in place of magnetic amine microspheres. In 140 mM PB, 93% of the rRNA bound to the microspheres. In a solution of 100 mM PB + 1M NaCl, 83% was bound.

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Example 15

Use of Magnetic Quaternary Ammonium Microspheres
to Separate rRNA

Magnetic quaternary ammonium microspheres were
5 synthesized to further study the method of the invention
with other polycationic supports. Magnetic amine
microspheres (BioMag M4100) were purchased from Advanced
Magnetics, Inc. Iodomethane was from Aldrich Chemical
10 Co., Milwaukee, WI, USA, and 2,6-lutidine was from Sigma
Chemical Co., St. Louis, MO, USA. Other materials were
reagent grade.

Magnetic amine microspheres (250 mg, 5.0 ml)
were diluted into 10 ml of water. The microspheres were
separated magnetically and washed by suspension in 20 ml
15 of 50% (v/v) ethanol/water followed by magnetic
separation. This wash process was repeated two times
with 20 ml of absolute ethanol. The washed microspheres
were then resuspended in 20 ml absolute ethanol.
Iodomethane (250 μ l, 4 mmoles), and 2,6-lutidine (24.5
20 μ l, 210 μ moles) were added with stirring; stirring was
continued overnight at room temperature. The
microspheres were then removed magnetically, washed four
times with 20 ml water as described above, and
resuspended in 5 ml of water for storage at 4°C.

The magnetic quaternary amine microspheres
25 were then used to remove [³H]-rRNA and [³²P]
deoxyoligonucleotides from defined buffers to study the
ability of the support to distinguish RNA
polynucleotides from DNA oligonucleotides in solution.
30 The deoxyoligonucleotide was the 36mer described in
Example 8. All other materials were the same as those
described in Example 1.

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The binding was determined using the procedure described in Example 1 and the results are shown in Table 11.

Table 11

	<u>Reagent System</u>	<u>% [³H]-rRNA Bound</u>	<u>% [³²P]-DNA Bound</u>
5	0.1M PB, pH 5.7	99	---
	0.1M PB, pH 6.8	98	---
	0.1M PB, pH 7.8	96	---
	0.1M BB ^{13/} , pH 9.5	97	---
10	0.1M PB, pH 6.8	98	62
	0.15M PB, pH 6.8	97	57
	0.20M PB, pH 6.8	97	39
	0.30M PB, pH 6.8	89	6

Example 16

Use of Magnetic Poly-D-Lysine Functionalized Microspheres to Separate rRNA

In addition to the cationic derivatives described supra in Examples 14 and 15, magnetic microspheres were derivatized with poly-D-lysine.

Materials: Carboxyl terminated magnetic microspheres were purchased from Advanced Magnetics, Inc. (Biomag M4125, containing approximately 250 uequiv. of carboxyl groups per gram). Poly-D-lysine, average degree of polymerization 68 monomer units, was from Sigma Chemical Co., St. Louis, MO, USA. N,N'dicyclohexyl carbodiimide (DCC) was from Pierce Chemical Co., Rockford, IL, USA; and

^{13/} BB = Sodium Borate Buffer

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N-hydroxysuccinimide (NHS) was from Eastman-Kodak Co., Rochester, NY, USA.

5 Ten milliliters of a 20 mg/ml suspension of carboxyl terminated magnetic microspheres was transferred to a glass teflon lined screw cap test tube. The microspheres were separated magnetically and washed sequentially with 0.1 N NaOH (10 ml), 0.1 M EDTA (10 ml, pH 7), water (2 X 10 ml), 50 % dioxane/water (10 ml), and dry dioxane (3 X 10 ml). The microspheres were then resuspended in dry dioxane (10 ml) containing NHS (250 mg). Next, DCC was added (400 mg); the tube was covered with foil to exclude light; and the suspension was mixed by end over end rotation for 15 hours. After magnetic separation, the NHS-modified microspheres were washed sequentially with dioxane (3 X 10 ml), methanol (3 X 10 ml), and dry dioxane (3 X 10 ml), and resuspended in dry dioxane (10 ml). One milliliter of the resulting suspension of NHS-modified microspheres was separated magnetically, washed with dilute aqueous HCl (pH 4.3), and resuspended in a 0.5 ml solution of 0.2 M NaHCO₃/0.4 M NaCl/0.05% NaN₃ containing poly-D-lysine (7 mg). After 2 hours, an aliquot of a solution of 1 M ethanolamine HCl (pH 8.4, 100 µl) was added to cap unreacted NHS ester groups. The microspheres were separated magnetically, washed with water (2 X 1 ml) and resuspended in water (0.5 ml).

10 20 25

30 The poly-D-lysine functionalized magnetic microspheres were shown to exhibit binding discrimination between rRNA and a synthetic oligonucleotide (26 mer) as follows:

[³H]-labeled rRNA from E. coli and a [³²P]-end labeled synthetic oligonucleotide were obtained.

To a screw-cap 1.5 ml polypropylene tube was added poly-D-lysine functionalized microspheres (5 µl),

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buffer (0.1 M sodium phosphate, pH 6.8/0.75 M NaCl, 0.5 ml), and an aliquot from either of a solution of [³H]-rRNA or a solution of [³²P]-oligonucleotide. Resulting suspensions were mixed by vortexing repeatedly over a period of 10 minutes. The supernatants were removed by magnetic separation and transferred to vials containing scintillation fluid (5 ml, BetagelTM cocktail). Radioactivity was then quantified by scintillation counting:

[³²P]-oligonucleotide bound to microspheres: 0 - 3%
[³H]-rRNA bound to microspheres: 78 - 82%

Example 17

Use of Magnetic Amine Microspheres in a
Chemiluminescent Nonisotopic Assay

To demonstrate the ability of magnetic amine microspheres to be used as a separation support for a non-isotopic assay system, separation of hybrids formed using a synthetic deoxyoligonucleotide probe labeled with a chemiluminescent acridinium ester was studied. In addition to the materials listed in Example 1, a deoxyoligonucleotide probe (33-mer) specific for Chlamydia trachomatis rRNA was synthesized and labeled with a chemiluminescent acridinium ester (AE) as described in U.S. Patent App. Ser. No. 105,080 entitled "Acridinium Ester Labeling and Purification of Nucleotide Probes" filed October 2, 1987 by Arnold, et al., Chlamydia trachomatis rRNA was used and assay tubes were 12 x 75 mm polystyrene tubes. All other components were reagent grade.

The AE-labeled probe was hybridized to its

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target RNA (in this case, Chlamydia trachomatis) according to the following procedure:

Hybridization mixture

200 μ l hybridization buffer (0.1M lithium succinate, pH 5.2, 10% lithium dodecyl sulfate, 2 mM EDTA and 2 mM EGTA)
5 1 μ l RNA (10^{-3} μ g)
1 μ l AE-probe (0.125 pmol)

10 The control mixture was the same as the hybridization mixture except that it contained water instead of RNA. The mixtures were incubated 30 minutes at 60°C, 2 ml of separation solution containing 0.4M PB, pH 6.0, 5% (v/v) Triton X-100, 8% (wt/v) DIBSS and 2.5 mg of magnetic amine microspheres (BioMag M4100) was added, the mixtures were vortexed and incubated an additional 5 minutes at 60°C. The magnetic amine microspheres were then magnetically pulled to the side of the tube, the supernatant was decanted off, and the magnetic amine microspheres were washed 3 times with 2 mL 0.4M PB, pH 6.0, pre-warmed to 60°C (add wash buffer, vortex, magnetically separate, decant). The bound probe was then eluted from the magnetic amine microspheres by adding 300 μ l of elution buffer containing 0.2M PB, pH 6.0, 50% formamide, vortexing and incubating 5 minutes at 60°C.
15 The magnetic amine microspheres were magnetically pulled to the side of the tube, and the solution was transferred to a new assay tube. The chemiluminescence of each sample was then measured in a Berthold Clinilumat Model LB9502 (Wildbad, West Germany) by automatic injection of 200 μ l of 0.25 N HNO₃, 0.1% H₂O², followed after a one second delay by 200 μ l of 1N
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25
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NaOH and reading of chemiluminescence for 5 seconds (results given in "relative light units, or rlu").

RESULTS:

Control - 160 rlu
5 10^{-3} μ g rRNA - 3929 rlu

These results demonstrated that the magnetic amine microspheres can clearly separate hybridized from unhybridized probe in an assay system utilizing a non-isotopic label. Here we see a very low background (less than 0.01% of input rlu), and very distinct signal even at a very low concentration (10^{-3} μ g) of target RNA.

Example 18

15 Use of Magnetic Amine Microspheres in a
Chemiluminescent Nonisotopic Assay
with a Clinical Specimen

To demonstrate the ability of magnetic amine microspheres to be used as a separation support for a non-isotopic assay system in the presence of a clinical specimen, the AE-probe described in Example 17 was used to detect a dilution series of target rRNA in clinical media. In addition to the materials listed in Example 17, throat swab material was obtained from volunteers and placed in 3% lithium dodecylsulfate, 30 mM PB (pH 6.8), 1 mM EDTA and EGTA (this will subsequently be referred to simply as "throat swab"). AE-probe was hybridized in throat swab material to decreasing amounts of its target rRNA (in this case Chlamydia trachomatis) according to the following procedure:

30 50 μ l throat swab
6 μ l 4.8 M PB (pH 4.7)

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2 μ l AE-probe (1 pmole)

2 μ l RNA (3×10^{-3} , 3×10^{-2} , 3×10^{-1} μ g)

The control mixture was the same as the hybridization mixture except that it contained water instead of RNA. 5 The mixtures were incubated 60 minutes at 60°C, and one-third of each was then separated, washed and eluted as described in Example 17.

RESULTS:

10	Control	-	4,049 rlu
	10^{-3} μ g rRNA	-	9,500 rlu
	10^{-2} μ g rRNA	-	61,000 rlu
	10^{-1} μ g rRNA	-	657,000 rlu

These results demonstrate that the magneticamine microspheres can clearly separate hybridized from unhybridized probe in the presence of clinical specimen in an assay system utilizing a non-isotopic label. 15 The control is higher here than in Example 17, at least in part, due to a larger amount of probe used in this sample.

20

Example 19

Synthesis of Additional Polycationic Supports

The following supports were synthesized to further demonstrate the use of cationic supports in hybrid separations.

25

(a) Spermine latex microspheres:

Spermine was chosen in this example because it is a polyamine whose cations are spaced to correspond to the polynucleotide's anions. Latex amine microspheres

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(2.5% solids, 1 μ mean diameter, 0.125 mequiv./g) was purchased from Polysciences, Inc., Warrington, PA, USA. 1,4-butanediol, diglycidyl ether and spermine were from Aldrich Chemical Co., Milwaukee, WI, USA. A Millititer 96 filtration unit and 0.22 μ DuraporeR filters were gifts from Millipore Filter Corporation, Bedford, MA, USA. All other materials were reagent grade.

The microspheres were first activated with 1,4-butanediol diglycidyl ether: 25 mg (1 ml) of latex amine microspheres was filtered onto a 0.22 μ DuraporeR filter. The microspheres were then resuspended in 150 μ l of 0.6 N NaOH containing 2 mg/ml NaBH₄, and the slurry was transferred to a glass test tube to which 1,4-butanediol diglycidyl ether (150 μ l) was added slowly with swirling. The mixture was vortexed briefly every 10 minutes for a period of 1 hour followed by the addition of 1 ml of water. The microspheres were then filtered as described above and washed with water (2 ml).

The activated microspheres were then reacted with spermine. The epoxide modified microspheres from the reaction above were suspended in 1 ml of 0.1 M Na₂CO₃, pH = 11.6, and 75 μ l of spermine (warmed to 50°C) was added with mixing. After reacting 12 hours at room temperature, the microspheres were filtered, washed with water (2 ml), and resuspended in water (800 μ l).

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5 (b) Synthesis of Tris (2-aminoethyl) Amine Latex Microspheres ("Tris-Latex"):

10 Tris (2-aminoethyl) amine was purchased from Aldrich Chemical Co., Milwaukee, WI, USA. This compound was chosen because its cations are spaced roughly the same distance apart as polynucleotide anions.

15 Tris-(2-aminoethyl) amine (50 μ l) was added to activated microspheres according to the procedure described in part (a) of this example, supra.

20 (c) Synthesis of Tris (2-Aminoethyl) Amine Sepharose 4B ("Tris-Sepharose"):

25 Tresyl-activated sepharose 4B was purchased from Pharmacia Fine Chemicals AB, Uppsala, Sweden. Freeze-dried tresyl-activated Sepharose 4B (1 g) was washed on a 0.22 μ Durapore R (Millipore Corp., Bedford, MA, USA) filter with 1 mM HCl (200 ml) over a period of 45 minutes. The gel was then transferred to a 15 ml polypropylene screw-cap tube containing 5 ml of 0.1 M NaHCO₃ (pH 8)/0.5 M NaCl, and 200 μ l of tris (2-aminoethyl) amine. The reaction was mixed at room temperature by slow end over end rotation for a period of 2 hours. The gel was recovered by filtration and washed with 10 ml of 0.1 M NaHCO₃ (pH 8)/0.5 M NaCl, followed by 50 ml of 0.1 M Tris (pH 8). The derivatized gel was then transferred to a 15 ml screw-cap tube and washed further in 10 ml of 0.1 M Tris (pH 8) by end over end rotation for 4 hours. The gel was filtered and washed with 10 ml of 0.1 M sodium acetate (pH 4)/0.5 M NaCl, followed by 10 ml of 0.1 M Tris (pH 8)/0.5 M NaCl. This wash cycle was repeated two times. The gel was then suspended for storage at 4°C in 5 ml 0.1 M Tris (pH 8)/0.5 M NaCl/0.02% sodium azide.

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(d) Synthesis of Tris-(2-Aminoethyl) Amine Acrylic Microspheres ("Tris-Acrylic Microspheres"):

5 Materials: Tosyl-Activated Acrylic Microspheres were purchased from Kirkegaard & Perry Labs, Inc., Gaithersburg, MD, USA (mean particle size 3 μ).

10 One milliliter of activated microspheres (10% suspension) was transferred to a screw-cap 1.5 ml polypropylene tube. The microspheres were separated by centrifugation at 10,000 rpm for 5 minutes in a Tomy-Seiko Microcentrifuge (Model MR-15A, Tokyo, Japan); the supernatant was removed. Next, 0.1 M NaHCO₃ (0.9 ml) and tris-(2-aminoethyl) amine (0.1 ml) were added, and the microspheres were resuspended by vortexing. The contents of the tube were mixed by end over end rotation for 16 hours. The amine-modified microspheres were removed by centrifugation, washed with water (5 X 1 ml), and resuspended in 20 mM sodium phosphate buffered saline containing 0.02% NaN₃ (1 ml).

15

20 (e) Functionalization of a Hydrophilic Polyurethane Based Membrane with Tris-(2-Aminoethyl) Amine and with Poly-D-Lysine ("Tris-Polyurethane Membrane" and "Poly-D-Lysine Polyurethane Membrane"):

25 HPI Affinity Membrane (hydrophilic polyurethane base) was obtained from Amicon Corp., Danvers, MA, USA. The approximate pore size and thickness of this membrane were given as 1.2 micron and twelve thousandths of an inch, respectively.

30 2-fluoro-1-methylpyridinium phosphate (FMP) was purchased from Aldrich Chemical Co., Milwaukee, WI, USA; poly-D-lysine (average 68 monomers per molecule) was from Sigma Chemical Co., St. Louis, MO, USA.

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The available hydroxyl groups on the membrane were activated with FMP by a modification of a procedure reported by T.T. Ngo, Biotechnology, 4, 134 [1986]. 5 1 cm squares of the membrane were transferred to a 15 ml flat bottom screw cap vial and washed twice with dry acetonitrile (5 ml). The squares were then suspended in dry acetonitrile (4 ml) containing redistilled triethylamine (80 μ l); and a solution of FMP (200 mg) in dry acetonitrile (10 ml) containing triethylamine (100 μ l) was added dropwise with swirling. The contents of 10 the vial were then swirled on a rotating platform for one hour, after which the solution was removed and the membrane pieces were washed sequentially with acetonitrile (2 X 5 ml), acetone (5 ml), 50:50 acetone/aqueous 5 mM HCl (5 ml) and aqueous 5 mM HCl (5 ml). The FMP activated membrane squares were then divided between two glass vials and treated with 5 ml of 15 0.5 M NaHCO₃ containing either tris-(2-aminoethyl) amine (100 μ l) or poly-D-lysine (50 mg). The contents of each vial were mixed on a rotating platform for 15 hours. Finally, the amine-derivatized membrane pieces were 20 washed sequentially with 1 M NaCl (3 X 5 ml), 0.1 M sodium phosphate buffer pH 7 (3 X 5 ml) and water (3 X 5 ml), and were then blotted dry on a sheet of 3MM paper (Whatman Ltd., Maidstone, England) and stored dry at room 25 temperature.

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Example 20

Separation of Nucleic Acids with Spermine
Latex Microspheres

5 Spermine latex microspheres were prepared as described in Example 19. Stock [³H] rRNA was from E. coli as described, supra. [-³²P] ATP was from New England Nuclear Research Products, Inc., Boston, MA, USA; and T4 polynucleotide kinase was from Bethesda Research Labs, Inc., Gaithersburg, MD, USA. Betagel™ (liquid scintillation cocktail) was from WestChem, San Diego, CA.

10 Probe synthesis and labeling: a deoxynucleotide with the sequence "5'-GGCGTTACCCCACCTACTAGCTAAT-3'" was produced using an Applied Biosystems, Inc. Model 380A DNA Synthesizer (Foster City, CA, USA) using standard phosphoramidite chemistry¹⁴. (This sequence is complementary to the bases 235-260 of the 23S

15 subunit of the E. coli ribosome). This oligomer was labeled on the 5'-end using [-³²P] ATP and T4-polynucleotide kinase according to the procedure of Maxam and Gilbert (Proc. Natl. Acad. Sci. U.S.A., 74, 560 [1977]).

20 [³H]-rRNA and [³²P]-DNA immobilizations: 0.5 ml of test buffer, 20 µl of spermine latex microspheres and 0.5 µl of either [³H]-rRNA (14,000 CPM) or [³²P] DNA oligomer (15,000 CPM) were mixed and incubated at 50°C for five minutes. The microspheres were then pelleted by centrifugation at 13,000 RPM for two minutes in a Tomy Seiko Model MR-15A Microcentrifuge (Tokyo, Japan). The supernatants were removed and added to 15 ml of

¹⁴/ Probe sequence patent pending.

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Betagel™ in 20 ml polypropylene tubes. The amount of [3H] or [32P] in each sample was determined using a Nuclear Chicago scintillation counter.

The results are shown in Table 12. At concentrations between 200 mM and 400 mM PB, the sperm latex microspheres maximally removed target polynucleotides and minimally bound probe oligonucleotides. The preferential selection continued into higher buffer concentrations.

10

Table 12

	<u>Buffer Strength</u>	% [³ H]-rRNA Bound to <u>Spermite Latex</u>	% [³² P] - D N A Oligomer Bound <u>to Spermite Latex</u>
		10 mM PB	50 mM PB
15	50 mM PB	98.4%	95.7%
	100 mM PB	97.6%	92.9%
	200 mM PB	95.2%	41.2%
	400 mM PB	93.5%	6.5%
	600 mM PB	57.3%	-----
	800 mM PB	78.5%	-----

Example 21

Separation of Nucleic Acids with Tris-Latex Microspheres

Tris-latex microspheres were prepared as described in Example 19, supra. Other materials are described in Example 20.

Manipulations were as described supra using Tris-latex microspheres in place of Spermite latex microspheres. Results are shown in Table 13. As with spermite latex, the Tris-latex microspheres showed

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optimum selection of polynucleotide targets over oligonucleotide probes at 200 mM to 400 mM PB.

Table 13

	<u>Buffer Strength</u>	<u>% [³H]-rRNA Bound to Tris-Latex</u>	<u>% [³²P]-DNA Bound to Tris-Latex</u>
5	10 mM PB	92.0%	----
	50 mM PB	97.2%	64.6%
	100 mM PB	97.4%	69.4%
	200 mM PB	98.5%	16.5%
10	400 mM PB	96.8%	8.3%
	600 mM PB	70.3%	----
	800 mM PB	79.4%	----

Example 22

Separation of Nucleic Acids with Tris-Sepharose

15 Tris-sepharose was prepared as described in Example 19. Other materials are described in Example 20. Tris-sepharose (100 μ l) was added to 0.5 ml of 0.3M PB and 0.5 μ l of either [³H]-rRNA or [³²P] DNA oligomer. The contents were incubated at 60° for 15 minutes with frequent swirling to suspend the gel. The gel was pelleted by centrifugation at 13,000 RPM for 30 sec. and the supernatant was removed and transferred to a 20 ml polypropylene vial containing 15 ml of Betagel™. The gel was washed two times with 0.5 ml of 0.3 M PB; the washings were separated by centrifugation and transferred to polypropylene vials containing Betagel™ as described above. Finally, the gel was suspended in 0.5 ml of 0.3 M PB and likewise transferred to a polypropylene vial containing 15 ml of Betagel™. The amount of [³H] or [³²P] in each sample was determined by

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scintillation counting as described supra. The results in Table 14 show the preferential binding of RNA polynucleotide over DNA oligonucleotide.

Table 14

5	<u>Sample</u>	<u>Percent Bound to Gel</u>
	[³ H]-rRNA	75.1%
	[³ H]-rRNA	80.6%
	[³² P]-DNA (26-mer)	0.54%
	[³² P]-DNA (26-mer)	0.71%

10

Example 23

Separation of Nucleic Acids with
Tris-Acrylic Microspheres

15 Binding discrimination of the tris-(2-aminoethyl) amine derivatized acrylic microspheres (described in Example 19, supra) for rRNA versus a synthetic oligonucleotide was demonstrated.

20 [³H]-rRNA from E. coli and a [³²P] end-labeled oligonucleotide (26 mer) are described in Example 20. To a 1.5 ml screw cap polypropylene tube were added: tris-(2-aminoethyl) amine modified microsphere suspension (7 μ l), sodium phosphate buffer (pH 6.8, 200 μ l, ranging from 0.1 - 0.4 M), and either of a solution of [³H] rRNA or a solution of [³²P]-labeled oligonucleotide (1 μ l). The suspension was heated at 25 50 C for 10 minutes, and then was transferred to a well of a microtiter filtration manifold (0.22 μ pore size, Millipore, Inc., Bedford, MA, USA). Vacuum was applied, and the microspheres were washed on the filter with the same buffer (200 μ l). The vacuum was released and the

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microspheres were then resuspended in 20 mM sodium phosphate buffered saline (200 μ l) and transferred to a vial containing scintillation cocktail (10 ml, BetagelTM). Radioactivity remaining bound to the microspheres was quantitated by scintillation counting. The data is summarized in Table 15.

Table 15

	<u>Buffer</u>	PERCENT BOUND	
		[³ H]-rRNA	[³² P]-DNA Probe
10	0.1 M PB	74%	18%
	0.2 M PB	71%	7%
	0.3 M PB	61%	6%
	0.4 M PB	47%	7%

15

Example 24
Separation of Nucleic Acids with Poly-D-Lysine
Polyurethane Membrane and Tris-Polyurethane Membrane

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The poly-D-lysine modified membrane pieces described in Example 19, supra, were shown to exhibit similar binding discrimination for rRNA versus a synthetic oligonucleotide (19 mer) as has been already described, supra.

25

[³H]-rRNA from E. coli and a [³²P] end-labeled oligonucleotide (19 mer) were used. Pieces of amine modified membrane were positioned on top of three sheets of 3MM paper (Whatman, Ltd., Maidstone, England) in a slot blotting device (J.M. Specialty Parts, San Diego, CA, USA). 1 μ l aliquots of either [³H]-rRNA or [³²P]-labeled oligonucleotide were diluted into 200 μ l of either Buffer A (0.1 M sodium phosphate (pH 6.8)/0.15 M NaCl/0.02% SDS/0.1% Triton X-100) or Buffer B (0.1 M

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sodium phosphate (pH 6.8)/0.45 M NaCl/0.02% SDS/0.1% Triton X-100. The resulting solutions were then loaded into appropriate wells of the slot blotter and passed through the pieces of amine modified membrane by capillary action. The membrane pieces were then removed from the slot blotter, washed with the same buffer (either Buffer A or Buffer B, 2 X 2 ml), and transferred to vials containing 5 ml of scintillation cocktail (BetagelTM). Radioactivity bound to the membrane pieces was quantitated by scintillation counting; the results are given in Table 16.

Table 16

	<u>Amine Modifier</u>	<u>Buffer</u>	<u>Percent bound</u>	
			<u>[³H]-RNA</u>	<u>[³²P]-DNA Probe</u>
15	Poly-D-lysine	A	49%	1%
	Poly-D-lysine	B	31%	0.4%
	Tris-(2-aminoethyl)amine	A	8%	0.3%
10	Tris-(2-aminoethyl)amine	B	8%	0.2%

Example 25

Use of Tris-Latex Microspheres with a Biotinylated DNA-Probe in a Colorimetric Assay

To determine the feasibility of the method of the invention for a colorimetric hybridization assay, tris latex microspheres were used in conjunction with a biotin labeled DNA probe to *Mycoplasma pneumoniae* rRNA:

(a) Synthesis of a biotin labeled DNA oligomer complementary to a region of *Mycoplasma pneumoniae* rRNA:

Materials: Biotinyl-E-Aminocaproic acid N-hydroxysuccinimide ester (Biotin-X-NHS) was purchased from Calbiochem-Behring Corp., San Diego, CA, USA.

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5-Allylamine UTP, terminal deoxynucleotide transferase, and 5x tailing buffer were products of Bethesda Research Laboratories, Gaithersburg, MD, USA. Bio-Gel P-60 (100-100 mesh) was from Bio-Rad Laboratories, Richmond, CA, USA; and Sephadex G-25 (medium) was from Pharmacia Fine Chemicals, Piscataway, NJ. Other materials used are described in Examples 19 and 20, supra. A deoxyribonucleotide probe 36 nucleotides in length, complementary to a sequence present in the 16S subunit of rRNA from Mycoplasma pneumoniae, was used. This oligomer was labeled on the 5'-end with [³²P] as described in Example 20.

15 Tailing reaction with allylamine UTP: 9 pmol of [³²P]-oligomer was reacted with 0.1 mM allylamine UTP and 40 units of terminal deoxynucleotide transferase in 50 μ l of 1 x tailing buffer at 37°C for 1 hour. The tailed oligomer was purified on a Bio-Gel P-60 column (.7 x 10 cm), eluting with 0.1 M PB/2 mM EDTA. The oligomer was then desalting by passing it through a Sephadex G-25 column and eluting with 0.2M triethylammonium bicarbonate (TEAB, pH 8).

20 Reaction with biotin-X-NHS: The allylamine UTP tailed oligomer, dissolved in 150 μ l of 0.1 M NaHCO₃ (pH 8.8)/0.02% SDS, was treated four times with 10 μ l of biotin-X-NHS (30 mM stock in DMSO) at 60 minute intervals. The biotinylated oligomer was purified on a column of Sephadex G-25 (0.7 x 10cm) eluting with 0.1 M PB/2 mM EDTA/0.02% SDS.

25 (b) Immobilization of the biotinylated DNA probe-rRNA Hybrid on "Tris-Latex": A stock solution of rRNA (1 ug/ μ l) from Mycoplasma pneumoniae was used. A

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suspension of tris-latex was prepared as described in Example 19. Other materials were reagent grade.

Hybridization reaction mixtures were as follows:

5 Hybrid: 1 μ l rRNA (1 μ g), 10 μ l biotinylated DNA probe (0.1 pmol), 0.4 μ l 1% SDS, 2.4 μ l 1 M PB, and 6.2 μ l H₂O totaling 20 μ l.

10 Probe Control: 10 μ l biotinylated DNA probe (0.1 pmol), 0.4 μ l 1% SDS, 2.4 μ l 1 M PB, and 7.2 μ l H₂O totaling 20 μ l.

15 The hybridization mixtures were incubated at 60°C for one hour followed by the addition of 0.5 ml of 0.4 M PB/0.02% SDS/1.0% Triton X-100 as well as 10 μ l tris-latex (aqueous suspension). The contents were mixed and incubated at 60°C for 5 minutes. The tris-latex microspheres were pelleted by centrifugation at 13,000 RPM for 2 minutes. The amount of [³²P] remaining bound to the microspheres was determined by scintillation counting using Cerenkov radiation.

20 In the hybrid experiment, 61% of the total radioactivity bound to the tris latex, while in the probe control only 5% bound. This reflects a 56% hybridization in the former experiment.

25 (c) The biotinylated DNA probe-rRNA hybrid was non-isotopically detected on Tris-Latex:

30 Reagents for the preparation of an avidin-alkaline phosphatase complex were purchased from Vector Laboratories, Burlingame, CA, USA. Bovine serum albumin (BSA), nitro blue tetrazolium salt (NBT), 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and Tween-20 were products of Sigma Chemical Co., St. Louis, MO, USA. Other materials are described in Examples 19 and 20.

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Two samples of Tris-latex incubated either with biotinylated DNA probe/rRNA hybrid or with biotinylated DNA probe alone (as described in this example, supra) were filtered on a Millititer 96 filtration apparatus employing Durapore R (Millipore Corp., Bedford, MA, USA) filters. Each sample of filtered solids was resuspended in 200 μ l of 3% BSA in 0.1 M Tris (pH 7.5)/0.1 M NaCl/3 mM MgCl₂/0.05% Tween-20, transferred to a 1.5 ml screw-cap polyethylene tube, and incubated at 44°C for 1 hour. The samples were then filtered as described above, and the filtered solids were resuspended in 200 μ l of streptavidin-alkaline phosphatase complex (prepared in phosphate-buffered saline according to the manufacturer's recommendations). After 10 minutes, the samples were filtered and washed two times with 200 μ l of 0.1 M Tris (pH 7.5)/0.1 M NaCl/3 mM MgCl₂/0.05% Tween-20, and then once with 200 μ l of 0.1 M Tris (pH 9.5)/0.1M NaCl/50 mM MgCl₂. The filtered solids were then resuspended in 200 μ l of 0.1 M Tris (pH 9.5)/0.1 M NaCl/50 mM MgCl₂ containing 0.33 mg/ml (NBT) and 0.17 mg/ml (BCIP). Color development was allowed to proceed for 10 minutes in the dark. The latex particles were then removed by filtration and washed two times with 0.1 M NaCl 3 mM MgCl₂/0.05% Tween-20.

A blue precipitate, deposited onto the surface of the Tris-latex microspheres, was easily visible for the hybrid sample within about 2 minutes. This was clearly distinguishable from the very faint color development observed for the sample with biotinylated probe alone.

Example 26

Use of Tris-Sepharose with a Biotinylated Probe

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in a Colorimetric Non-Isotopic Assay

The hybridization system of Example 25 was used with Tris Sepharose gel instead of tris-latex microspheres.

- 5 (a) The hybridized biotinylated probe was immobilized on Tris-Sepharose:

Small columns containing 0.1 ml of Tris-Sepharose (prepared as described in Example 19) were prepared using 1 cc Tuberculin syringes. Hybridization mixtures (prepared as described in Example 25(b) were dissolved in 0.5 ml of 0.25 M PB/0.02% SDS/1.0% Triton X-100, and passed dropwise through the tris-sepharose column over a 5 minute period. The columns were then washed two times with 0.5 ml of the same buffer. The amount of [³²P] remaining bound to the column was determined by scintillation counting as described in Example 25(b). Tris-sepharose exhibited particularly precise selection of hybrid over probe. Of the hybrid, 81% was bound to Tris Sepharose while 0.0% of the probe control was bound. (The amount of [³²P] remaining bound in the probe control was not measurable above background.

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(b) The biotinylated probe was non-isotopically detected.

5 The materials were as described in Example 21(c). Small columns of Tris-sepharose were prepared and treated with hybridization reaction mixtures as described in this example, supra. The avidin-alkaline phosphatase complex (0.5 ml, prepared as described in Example 25(c)) was then passed dropwise through the column. After 10 minutes, the remaining avidin-alkaline phosphatase solution was pushed out under slight positive pressure. The gel was washed two times with 0.5 ml of 0.1 M Tris (pH 7.5)/0.1 M NaCl/3 mM MgCl₂ and one time with 0.1 M Tris (pH 9.5)/0.1 M NaCl/50 mM MgCl₂. NBT/BCIP dye reagent (0.5 ml, see Example 25) 10 was then passed slowly through the gel. After 10 minutes, the remaining reagent was pushed out, and the gel was washed two times with 0.5 ml of 0.1 M Tris (pH 7.5)/0.1 M NaCl/3 mM MgCl₂.

15

20 Blue color development was visible for the hybrid sample within about 2 minutes. This was easily distinguishable from the sample with probe alone, which was only faintly blue after the 10 minute period of color development. Note: This support did not require the "capping" step with 3% BSA as was needed with the former support (Example 25(c)).

25

Example 27

Use of Tris-Sepharose with an Alkaline Phosphatase Labeled DNA Probe in a Colorimetric Assay

30 The utility of the cationic supports described in Example 19 was further demonstrated in a colorimetric hybridization assay employing an alkaline phosphatase/deoxyoligonucleotide probe conjugate.

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Materials: An alkaline-phosphatase labeled synthetic oligonucleotide (26 mer), complementary to rRNA from E. coli (hereafter referred to as the "target rRNA"), was prepared by a modification of a procedure described by E. Jablonski *et al.*, Nucl. Acids Res., Vol. 14, p. 6115 (1986). The oligonucleotide sequence was chosen to exhibit minimal cross hybridization to rRNA from Candida albicans (referred to hereafter as "non-target rRNA"). Target and non-target rRNA was isolated and purified. NBT and BCIP dyes have been described in Example 25, *supra*. Other materials were reagent grade.

Hybridization cocktails were prepared in 1.5 ml polypropylene microcentrifuge tubes by adding the oligonucleotide-alkaline phosphatase conjugate (10 μ l, 1 pmol), dilutions of either target or non-target rRNA (1 μ l, 1 - 0.0001 μ g), 4.8 M sodium phosphate buffer (2 μ l, pH 6.8), sodium dodecyl sulfate (0.4 μ l, 1% solution v/v), and sterile water (13.6 μ l): total volumes were 20 μ l. These cocktails were hybridized at 50°C for 30 minutes. Next, 0.3 M sodium phosphate (0.5 ml, pH 6.8) and tris-sepharose (about 30 μ l bed volume) were added, and the contents were mixed by shaking in a water bath at 50°C for 10 min. The tubes were then spun briefly in a microcentrifuge and the supernatants were drawn off with a pasteur pipette. The tris-sepharose pellets were then washed sequentially with 0.3 M sodium phosphate buffer (3 X 0.5 ml) followed by 50 mM Tris HCl (pH 8)/0.1 M NaCl/1 mM MgCl₂/0.1 mM ZnCl₂ (2 X 0.5 ml). The pellets were then resuspended in 300 μ l of a solution of 0.1 M Tris HCl (pH 9.5)/0.1 M NaCl/50 mM MgCl₂ containing NBT (0.33 mg/ml) and BCIP (0.25 mg/ml) and incubated at 42°C for four hours. Blue-violet

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coloration was visible on tris-sepharose pellets resulting from hybridization reactions with as little as 0.001 µg of target rRNA. No coloration was evident on tris-sepharose pellets from the controls with as much as 1.0 µg of non-target rRNA.

Example 28

Use of Poly-D-Lysine Polyurethane Membrane with
an Alkaline Phosphatase Labeled DNA Probe in a
Colorimetric Assay

In this experiment, the utility of a cationic membrane as the separation support in a colorimetric assay was demonstrated.

Hybridization reactions were performed as described in Example 27. To these reactions was added 300 µl of dilution buffer (0.1 M sodium phosphate (pH 6.8)/0.45 M NaCl/0.02% SDS/0.1% Triton X-100). The resulting solutions were passed through pieces of poly-D-lysine membrane by capillary action using a slot blotter apparatus as described in Example 24. The membrane pieces were then removed from the slot blotter and washed with dilution buffer (2 X 2 ml) and assay buffer (2-amino-2-methylpropanediol HCl (pH 10.2)/0.1 M NaCl, 2 X 4 ml). Then, the membrane pieces were immersed in assay buffer (10 ml) containing NBT (0.33 mg/ml) and BCIP (0.25 mg/ml). After 10 minutes, slots treated with hybridization reactions containing 1 µg of target rRNA gave dark blue-violet coloration, whereas controls containing 1 µg of non-target rRNA gave no coloration.

This colorimetric assay did not require the prehybridization, fixation and blocking steps typically

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associated with slot-blot assays and therefore represents a significant improvement over the existing art.

Example 29

5 Use of Poly-D-Lysine Polyurethane Membrane in
 a Chemiluminescent Non-Isotopic Assay

10 The utility of the cationic membrane separation support described in Example 19 was demonstrated in a chemiluminescent nonisotopic hybridization assay employing an acridinium ester (AE) labeled DNA probe.

15 The methods for generation and detection of chemiluminescent acridinium ester labeled oligonucleotide probes were performed as described in U.S. Patent App. Ser. No. 105,080 entitled "Acridinium Ester Labeling and Purification of Nucleotide Probes", filed October 2, 1987, incorporated herein by reference. An acridinium ester (AE) labeled oligonucleotide (26 mer) complementary to a sequence in rRNA from *E. coli* was prepared by incorporating a single acridinium ester per probe molecule. Other materials and reagents are described in Examples 19 and 27, supra.

20 Hybridization cocktails were prepared in 1.5 ml polypropylene microcentrifuge tubes: AE-labeled probe (2 μ l, 1.5×10^6 relative light units, "RLU's"), 1% SDS (v/v, 1 μ l), 1 M sodium phosphate buffer (pH 4.9, 5 μ l), and either of target or non-target rRNA (1 μ g/ μ l, 1 μ l), total reaction volume 9 μ l. The contents of each tube were incubated at 60 °C for 30 minutes. Separation buffer (0.6 ml, 0.1 M sodium phosphate pH 6.8/0.15 M NaCl/0.02% SDS/ 0.1% Triton X-100) was added, and the resulting solutions were transferred by capillary action

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onto pieces of poly-D-lysine modified membrane using a slot blotter apparatus as described supra. The pieces of membrane below each slot were individually washed in separation buffer (3 X 2 ml), and then were cut in 0.25 cm² pieces and transferred to 12 X 75 mm polypropylene tubes (Sarsted, West Germany) containing 200 µl of water. The contents of each tube were quantified for relative chemiluminescence as referenced supra. Specifically, a Clinilumat Model LB 9502 Luminometer was used (Berthold, Wildbad, West Germany) with a two injection cycle: Injection #1- 0.25 M HNO₃/0.1% H₂O₂ (200 µl), Injection #2- 2 M potassium phosphate buffer (pH 13.2, 200 µl). Results are given in Table 17.

15

Table 17

<u>Sample Applied to Membrane</u>	<u>Relative Chemiluminescence</u>
Separation Buffer Only	27,291
Hybridization Reactions, Non-Target rRNA	21,807
	51,292
	18,961
Hybridization Reactions, Target rRNA	192,058
	286,842
	242,625

25

Example 30
Use of Tris-Sepharose in a Chemiluminescent
Non-Isotopic Hybridization Assay

Acridinium ester labeled oligonucleotide probe/rRNA hybridization reactions were run as described in Example 29. Aliquots from these reactions were

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transferred to clean 1.5 ml polypropylene microfuge tubes and diluted with 0.3 M sodium phosphate buffer (pH 6.8, 0.5 ml). Next, tris-sepharose (30 μ l bed volume, prepared as described in Example 19) was added, and the contents were mixed by gentle vortexing at room temperature for 10 minutes. After a brief spin in a microcentrifuge, the supernatants were removed and the resulting tris-sepharose pellets were washed with 0.3 M sodium phosphate (pH 6.8, 2 X 0.5 ml) and then suspended in 0.3 M sodium phosphate (pH 6.8, 0.5 ml). Aliquots of the resulting suspensions (0.4 ml) were transferred to 12 X 75 mm polypropylene tubes; and 30% hydrogen peroxide was added (0.5 μ l). Chemiluminescence was measured as described in Example 29 except that a single injection of 2 N NaOH (200 μ l) was used to initiate the chemiluminescent reaction. Results are given in Table 18.

Table 18

	<u>Sample Bound to</u>	<u>Relative Chemiluminescence</u>
20	<u>Tris-Sepharose</u>	
	Control (no target)	637
	Target (<u>E. coli</u> rRNA)	97,071
	Non-Target (<u>C. albicans</u> rRNA)	702

Those skilled in the art will appreciate that the methods and compositions described above can be used to purify nucleic acids and to detect target nucleotide sequences in DNA and RNA derived from a wide variety of sources including infectious agents such as bacteria, viruses and fungi; cancer cells; and cells which may yield information about genetic diseases such as sickle

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cell anemia. Accordingly, the scope of this invention will only be limited by reference to the appended claims.

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WE CLAIM:

1. A method for purifying nucleotide multimers in a solution containing contaminants comprising the steps:

- 5 a. contacting the solution with a polycationic solid support to non-covalently bind the multimer to the support without significant binding of the contaminants and;
- 10 b. segregating the support and bound multimers from the solution.

2. A method according to Claim 1 wherein the support is treated to recover the multimer.

15 3. A method according to claim 2 wherein the support is treated with an elution solution to desorb the multimers followed by separation of the elution solution and solid support then separated.

20 4. A method according to claim 3 wherein the elution solution is a member of the group consisting of phosphate, pyrophosphate, tripolyphosphate, phytic acid and 50% formamide.

5. A method according to Claim 3 wherein the multimers are recovered from the elution solution.

6. A method according to Claim 4 wherein the multimers are recovered from the elution solution.

25 7. A method according to claims 1, 2, 3, 4, 5, or 6 wherein the solid support is washed after separation from the solution containing contaminants.

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8. A method according to Claim 7 wherein the solution containing the multimers and contaminants is a separation solution.

5 9. A method according to Claims 1, 2, 3, 4, 5, or 6 wherein the multimers are polynucleotides.

10 10. A method according to Claim 9 wherein the contaminants include smaller nucleotide multimers than the bound polynucleotides.

10 11. A method according to Claims 1, 2, 3, 4, 5 or 6 wherein the solid support is comprised of particles.

15 12. A method according to Claim 11 wherein the solid support is comprised of micron sized particles.

13. A method according to claims 1, 2, 3, 4, 5 or 6 wherein the solid support is comprised of fibers.

20 14. A method according to claims 1, 2, 3, 4, 5 or 6 wherein the solid support is a membrane.

15. A method according to Claim 12 wherein the solid support is comprised of magnetically responsive particles and segregation is facilitated by exposure to a magnetic field.

25

16. A method according to claim 13 wherein the solid support is comprised of micron sized magnetically

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responsive fibers and segregation is facilitated by exposure to a magnetic field.

5 17. A method according to claim 14 wherein the said membrane is magnetically responsive and segregation is facilitated by exposure to a magnetic field.

10 18. A method according to Claims 1, 2, 3, 4, 5 or 6 wherein the cationic charge is imparted by alkyl or aryl amines, guanidines or imines.

15 19. A method according to Claim 18 wherein the solid support is a metal oxide, glass, polyamide, polyester, polyolefin, polysaccharide, polyglycol, or polyaminoacid.

20 20. An assay for detecting a target nucleotide sequence in a test solution comprising the steps:

25 a. contacting a labelled nucleotide probe for the target sequence with said test solution under hybridizing conditions to form a hybrid between said nucleotide probe and said target in the test solution;

25 b. contacting a polycationic solid support with said test solution to effectuate formation of a bound phase and a free phase;

30 c. detecting said bound phase or said free phase as a qualitative or quantitative measure of said target in the test solution.

30 21. An assay for detecting a target

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nucleotide sequence in a polynucleotide comprising the steps:

- 5 a. adding to a solution containing polynucleotide suspected of containing the target sequence a nucleotide probe for the target sequence, under hybridizing conditions to form a hybrid between the probe and target sequence if present;
- 10 b. combining the polynucleotides, including any hybrids with the probe and unhybridized probe in a solution with a polycationic solid support;
- 15 c. segregating the solid support from the solution containing unhybridized probe; and
- 20 d. detecting the presence of any unbound probe or probe bound to the solid support as a quantitative or qualitative measure of the target polynucleotide sequence in said test solution.

22. An assay for detecting a target nucleotide sequence in a polynucleotide comprising the steps:

- 20 a. adding to a solution containing polynucleotide suspected of containing the target sequence a nucleotide probe for the target sequence, the probe being a smaller nucleotide multimer than the polynucleotides being assayed, under hybridizing conditions to form a hybrid between the probe and target sequence if present;
- 25 b. combining the polynucleotides, including any hybrids with the probe and unhybridized probe in a solution with a polycationic solid support to non-covalently bind the polynucleotides and hybrids with the probe without significant binding of the unhybridized probe;

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c. segregating the solid support from the solution containing unhybridized probe; and

5

d. detecting the presence of any probe bound to the solid support as an indication of the presence and/or amount of target sequence in polynucleotides bound to the support.

10

23. An assay for detecting a target nucleotide sequence in a polynucleotide comprising the steps:

15

a. adding to a solution containing polynucleotide suspected of containing the target sequence a nucleotide probe for the target sequence, under hybridizing conditions to form a hybrid between the probe and target sequence if present;

20

b. combining the polynucleotides, including any hybrids with the probe and unhybridized probe in a solution with a polycationic solid support;

25

c. segregating the solid support from the solution containing unhybridized probe; and

d. eluting the hybrid, the labelled probe or the label from the solid support using an elution solution; and

e. detecting the presence of any unbound probe or probe bound to the solid support as a quantitative or qualitative measure of the target polynucleotide sequence in said test solution.

30
24. A method according to claim 23 wherein the hybrid elution solution is a member of the group consisting of 50% formamide, phosphate, pyrophosphate, tripolyphosphate and phytic acid.

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25. A method according to Claim 22 wherein the polynucleotides being probed are at least about 3 times larger multimers than the probe.

5 26. A method according to Claim 22 wherein the polynucleotides being probed are at least about 5 times larger multimers than the probe.

10 27. A method according to Claim 20, 21, 22 or 23 wherein the probe is an analogue of DNA or RNA having a lower negative charge in the phosphate backbone than the phosphate diesters of native DNA or RNA.

28. A method according to Claim 27 wherein the analogue is a methyl phosphonate.

15 29. A method according to Claims 20, 21, 22 or 23, wherein the segregation of the solid support comprises removing it from the solution containing unhybridized probe.

20 30. A method according to claims 20, 21, 22 or 23 wherein the probe is an analogue of DNA or RNA having a lower negative charge in the phosphate backbone than the phosphate diesters of native DNA or RNA and wherein the segregation of the solid support comprises removing it from the solution containing unhybridized probe.

25 31. A method according to claims 20, 21, 22 or 23 wherein the probe is an analogue of DNA or RNA having a lower negative charge in the phosphate backbone than the phosphate diesters of native DNA or RNA and wherein the segregation of the solid support comprises

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removing it from the solution containing unhybridized probe and wherein the probe has a label which permits its detection.

5 32. A method according to Claim 30 wherein the solid support is washed prior to detection of the probe.

10 33. A method according to Claim 31 wherein the solid support is washed prior to detection of the probe.

15 34. A method according to Claims 20, 21, 22 or 23 wherein the probe has a label which permits its detection.

15 35. A method according to Claims 20, 21, 22 or 23 wherein the solid support is comprised of particles.

20 36. A method according to Claim 35 wherein the solid support is comprised of micron sized particles.

20 37. A method according to claim 20, 21, 22 or 23 wherein the solid support is comprised of fibers.

25 38. A method according to claim 20, 21, 22 or 23 wherein the solid support is a membrane.

25 39. A method according to Claim 36 wherein the solid support is comprised of magnetically responsive particles and segregation is obtained by exposing said particles to a magnetic field.

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40. A method according to claim 37 wherein the solid support is comprised of magnetically responsive fibers and segregation is obtained by exposing said fibers to a magnetic field.

5

41. A method according to claim 38 wherein the solid support is a magnetically responsive membrane and segregation is obtained by exposing said membrane to a magnetic field.

10

42. A method according to Claims 20, 21, 22 or 23 wherein the cationic charge is imparted by alkyl or aryl amines, guanidines or imines.

15

43. A method according to Claim 42 wherein the segregation of the solid support comprises removing it from the solution containing unhybridized probe.

44. A method according to Claim 43 wherein the solid support comprises micron sized particles.

20

45. A method according to claim 43 wherein the solid support comprises fibers.

46. A method according to claim 43 wherein the solid support is a membrane.

47. A method according to Claim 44 wherein the particles are an magnetically responsive and the segregation is obtained by exposing the particles to a magnetic field.

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48. A method according to Claim 45 wherein the fibers are an magnetically responsive and the segregation is obtained by exposing the particles to a magnetic field.

5

49. A method according to Claim 46 wherein the membrane is magnetically responsive and the segregation is obtained by exposing the particles to a magnetic field.

10

50. A method according to Claim 47 wherein the particles are metal oxide, glass, polyamide, polyester, polyolefin, polysaccharide, polyglycol or polyaminoacid.

15

51. A method according to Claim 48 wherein the fibers are metal oxide, glass, polyamide, polyester, polyolefin, polysaccharide, polyglycol or polyaminoacid.

20

52. A method according to Claim 49 wherein the membrane is metal oxide, glass, polyamide, polyester, polyolefin, polysaccharide, polyglycol or polyaminoacid.

25

53. An assay for detecting a target nucleotide sequence in a polynucleotide comprising the steps:

a. contacting a solution containing polynucleotides suspected of containing the target sequence with a polycationic solid support to non-covalently bind the polynucleotides to the solid support;

b. contacting the solid support and bound polynucleotides with a solution containing a

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nucleotide probe for the target sequence, the probe being a smaller nucleotide multimer than the polynucleotides being assayed, under hybridizing conditions to form a hybrid between the probe and target sequence, if present, without significant binding of the unhybridized probe to the solid support;

5 c. segregating the solid support from the solution containing unhybridized probe; and

10 d. detecting the presence of the hybrid, either qualitatively or quantitatively, as a measure of target sequence in polynucleotides.

15 54. An assay for detecting a target nucleotide sequence in a polynucleotide comprising the steps:

20 a. contacting a solution containing polynucleotides suspected of containing the target sequence with a polycationic solid support to non-covalently bind the polynucleotides to the solid support;

25 b. contacting the solid support and bound polynucleotides with a solution containing a nucleotide probe for the target sequence, the probe being a smaller nucleotide multimer than the polynucleotides being assayed, under hybridizing conditions to form a hybrid between the probe and target sequence, if present, without significant binding of the unhybridized probe to the solid support;

30 c. segregating the solid support from the solution containing unhybridized probe; and

d. eluting the hybrid, the labelled probe or the label from the solid support using an elution solution;

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e. detecting the presence of the hybrid, either quantitatively or qualitatively, as a measure of target sequence in polynucleotides.

5 55. A method according to Claim 53 or 54 wherein the polynucleotides being probed are at least about 3 times larger multimer than the probe.

10 56. A method according to Claim 53 or 54 wherein the polynucleotides being probed are at least about 5 times larger multimers than the probe.

15 57. A method according to Claim 53 or 54 wherein the probe is an analogue of DNA or RNA having a lower negative charge in the phosphate backbone than the phosphate diesters of native DNA or RNA.

15 58. A method according to Claim 53 wherein the analogue is a methyl phosphonate.

59. A method according to Claim 54 wherein the analogue is a methyl phosphonate.

20 60. A method according to Claim 58 wherein the segregation of the solid support comprises removing it from the solution containing unhybridized probe.

61. A method according to Claims 59 wherein the segregation of the solid support comprises removing it from the solution containing unhybridized probe and wherein the analogue is a methyl phosphonate.

25 62. A method according to Claim 60 wherein

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the solid support is washed prior to detection of the probe.

63. A method according to claim 61 wherein
the solid support is washed prior to detection of the
probe.

5 64. A method according to Claims 53 or 54
wherein the probe has a label which permits its
detection.

10 65. A method according to Claims 53 or 54
wherein the solid support is comprised of particles.

66. A method according to Claim 65 wherein
the solid support is comprised of micron sized
particles.

15 67. A method according to claims 53 or 54
wherein the solid support is comprised of fibers.

68. A method according to claims 53 or 54
wherein the solid support is a membrane.

20 69. A method according to Claim 66 wherein
the solid support is comprised of magnetically
responsive particles and segregation is obtained by
exposing the particles to a magnetic field.

25 70. A method according to Claim 67 wherein
the solid support is comprised of magnetically
responsive particles and segregation is obtained by
exposing the particles to a magnetic field.

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71. A method according to Claim 68 wherein the solid support is comprised of magnetically responsive particles and segregation is obtained by exposing the particles to a magnetic field.

5 72. A method according to Claims 53 or 54 wherein the cationic charge is imparted by alkyl or aryl amines, guanidines or imines.

10 73. A method according to Claim 72 wherein the segregation of the solid support comprises removing it from the solution containing unhybridized probe.

74. A method according to Claim 72 wherein the solid support comprises micron sized particles.

75. A method according to claim 72 wherein the solid support comprises fibers.

15 76. A method according to claim 72 wherein the solid support is a membrane.

20 77. A method according to Claim 74 wherein the particles are magnetically responsive and the segregation is obtained by exposing the particles to a magnetic field.

78. A method according to Claim 75 wherein the fibers are magnetically responsive and the segregation is obtained by exposing the fibers to a magnetic field.

25 79. A method according to Claim 76 wherein the membrane is magnetically responsive and the

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segregation is obtained by exposing the membrane to a magnetic field.

5 80. A method according to Claim 77 wherein the particles are metal oxide, glass, polyamide, polyester, polyolefin, polysaccharide, polyglycol or polyaminoacid.

10 81. A method according to Claim 78 wherein the fibers are metal oxide, glass, polyamide, polyester, polyolefin, polysaccharide, polyglycol or polyaminoacid.

15 82. A method according to Claim 79 wherein the membrane is metal oxide, glass, polyamide, polyester, polyolefin, polysaccharide, polyglycol or polyaminoacid.

20 83. A kit for assaying for a target nucleotide sequence in a solution containing polynucleotides suspected of containing the target sequence comprising:

a. a nucleotide probe for the target sequence; and

25 b. a polycationic support capable of separating polynucleotides according to their charge or length.

* 84. A kit for assaying for a target nucleotide sequence in a solution containing polynucleotides suspected of containing the target sequence comprising:

1 a. a nucleotide probe for the target sequence;

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b. a polycationic support capable of separating polynucleotides according to their charge or length; and

c. a hybrid elution solution.

5

85. A kit for assaying for a target nucleotide sequence in a solution containing polynucleotides suspected of containing the target sequence comprising:

10

a. a nucleotide probe for the target sequence, the probe being a smaller nucleotide multimer than the polynucleotides to be assayed; and

15

b. a polycationic support capable of non-covalently binding the polynucleotides and hybrids thereof with the probe without significantly binding the unhybridized probe.

20

86. A kit for assaying for a target nucleotide sequence in a solution containing polynucleotides suspected of containing the sequence comprising:

a. a nucleotide probe for the target sequence, the probe being a smaller nucleotide multimer than the polynucleotides to be assayed; and

25

b. a polycationic support capable of non-covalently binding the polynucleotides and hybrids thereof with the probe without significantly binding the unhybridized probe; and

c. a hybrid elution solution.

30

87. A kit according to Claim 84 or 86 wherein the hybrid elution solution is a member of the group consisting of 50% formamide, phosphate, pyrophosphate, tripolyphosphate and phytic acid.

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88. A kit according to Claim 85 wherein the probe is selected so that the polynucleotides being probed are at least about 3 times larger than the probe.

5 89. A kit according to Claim 85 wherein the polynucleotides being probed are at least about 5 times larger than the probe.

10 90. A kit according to Claim 83 or 84 or 85 or 86 wherein the probe is an analogue of DNA or RNA having a lower negative charge in the phosphate backbone than the phosphate diesters of native DNA or RNA.

91. A kit according to Claim 90 wherein the analogue is a methyl phosphonate.

15 92. A kit according to Claims 83 or 84 or 85 or 86 wherein the probe has a label to permit its detection.

93. A kit according to Claim 83 or 84 or 85 or 86 wherein the solid support is comprised of particles.

20 94. A kit according to Claim 93 wherein the solid support is comprised of micron sized particles.

95. A kit according to Claim 83 or 84 or 85 or 86 wherein the solid support is comprised of fibers.

25 96. A kit according to Claim 83 or 84 or 85 or 86 wherein the solid support is a membrane.

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97. A kit according to Claim 94 wherein the particles are magnetically responsive.

98. A kit according to claim 95 wherein the fibers are magnetically responsive.

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99. A kit according to claim 96 wherein the membrane is magnetically responsive.

100. A kit according to claims 83 or 84 or 85 or 86 wherein the cationic charge is imparted by alkyl or aryl amines, guanidines or imines.

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101. A kit according to Claim 100 wherein the solid support is comprised of particles.

102. A kit according to claim 100 wherein the solid support is comprised of fibers.

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103. A kit according to claim 100 wherein the solid support is a membrane.

104. A kit according to Claim 101 wherein the particles are magnetically responsive.

105. A kit according to Claim 102 wherein the particles are magnetically responsive.

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106. A kit according to Claim 103 wherein the particles are magnetically responsive.

107. A kit according to Claim 103 wherein the particles are a metal oxide, glass, polyamide,

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polyester, polyolefin, polysaccharide, polyglycol or polyaminoacid.

108. A kit according to Claim 104 wherein the fibers are a metal oxide, glass, polyamide, polyester, polyolefin, polysaccharide, polyglycol or polyaminoacid.

5 109. A kit according to Claim 105 wherein the membrane is a metal oxide, glass, polyamide, polyester, polyolefin, polysaccharide, polyglycol or polyaminoacid.

AMENDED CLAIMS

[received by the International Bureau
on 26 July 1988 (26.07.88);
original claims 19,20,23,48,50,51,52,
54,80-82,84,108 and 109 amended;
other claims unchanged (6 pages)]

responsive fibers and segregation is facilitated by exposure to a magnetic field.

17. A method according to claim 14 wherein the said membrane is magnetically responsive and segregation is facilitated by exposure to a magnetic field.

18. A method according to Claims 1, 2, 3, 4, 5 or 6 wherein the cationic charge is imparted by alkyl or aryl amines, guanidines or imines.

19. (amended) A method according to Claim 18 wherein the solid support is a metal oxide, glass, polyamide, polyester, polyolefin, polysaccharide, polyglycol, or polyamino acid.

20. (amended) An assay for detecting a target nucleotide sequence in a test solution comprising the steps:

a. contacting a labeled nucleotide probe for the target sequence with said test solution under hybridizing conditions to form a hybrid between said nucleotide probe and said target in the test solution;

b. contacting a polycationic solid support with said test solution to effectuate formation of a bound phase and a free phase;

c. detecting said bound phase or said free phase as a qualitative or quantitative measure of said target in the test solution.

21. An assay for detecting a target

- c. segregating the solid support from the solution containing unhybridized probe; and
- d. detecting the presence of any probe bound to the solid support as an indication of the presence and/or amount of target sequence in polynucleotides bound to the support.

23. (amended) An assay for detecting a target nucleotide sequence in a polynucleotide comprising the steps:

- a. adding to a solution containing polynucleotide suspected of containing the target sequence a nucleotide probe for the target sequence, under hybridizing conditions to form a hybrid between the probe and target sequence if present;
- b. combining the polynucleotides, including any hybrids with the probe and unhybridized probe in a solution with a polycationic solid support;
- c. segregating the solid support from the solution containing unhybridized probe; and
- d. eluting the hybrid, the labeled probe or the label from the solid support using an elution solution; and
- e. detecting the presence of any unbound probe or probe bound to the solid support as a quantitative or qualitative measure of the target polynucleotide sequence in said test solution.

24. A method according to claim 23 wherein the hybrid elution solution is a member of the group consisting of 50% formamide, phosphate, pyrophosphate, tripolyphosphate and phytic acid.

48. (amended) A method according to Claim 45 wherein the fibers are magnetically responsive and the segregation is obtained by exposing the particles to a magnetic field.

49. A method according to Claim 46 wherein the membrane is magnetically responsive and the segregation is obtained by exposing the particles to a magnetic field.

50. (amended) A method according to Claim 47 wherein the particles are metal oxide, glass, polyamide, polyester, polyolefin, polysaccharide, polyglycol or polyamino acid.

51. (amended) A method according to Claim 48 wherein the fibers are metal oxide, glass, polyamide, polyester, polyolefin, polysaccharide, polyglycol or polyamino acid.

52. (amended) A method according to Claim 49 wherein the membrane is metal oxide, glass, polyamide, polyester, polyolefin, polysaccharide, polyglycol or polyamino acid.

53. An assay for detecting a target nucleotide sequence in a polynucleotide comprising the steps:

a. contacting a solution containing polynucleotides suspected of containing the target sequence with a polycationic solid support to non-covalently bind the polynucleotides to the solid support;

b. contacting the solid support and bound polynucleotides with a solution containing a

nucleotide probe for the target sequence, the probe being a smaller nucleotide multimer than the polynucleotides being assayed, under hybridizing conditions to form a hybrid between the probe and target sequence, if present, without significant binding of the unhybridized probe to the solid support;

c. segregating the solid support from the solution containing unhybridized probe; and

d. detecting the presence of the hybrid, either qualitatively or quantitatively, as a measure of target sequence in polynucleotides.

54. (amended) An assay for detecting a target nucleotide sequence in a polynucleotide comprising the steps:

a. contacting a solution containing polynucleotides suspected of containing the target sequence with a polycationic solid support to non-covalently bind the polynucleotides to the solid support;

b. contacting the solid support and bound polynucleotides with a solution containing a nucleotide probe for the target sequence, the probe being a smaller nucleotide multimer than the polynucleotides being assayed, under hybridizing conditions to form a hybrid between the probe and target sequence, if present, without significant binding of the unhybridized probe to the solid support;

c. segregating the solid support from the solution containing unhybridized probe; and

d. eluting the hybrid, the labeled probe or the label from the solid support using an elution solution;

segregation is obtained by exposing the membrane to a magnetic field.

80. (amended) A method according to Claim 77 wherein the particles are metal oxide, glass, polyamide, polyester, polyolefin, polysaccharide, polyglycol or polyamino acid.

81. (amended) A method according to Claim 78 wherein the fibers are metal oxide, glass, polyamide, polyester, polyolefin, polysaccharide, polyglycol or polyamino acid.

82. (amended) A method according to Claim 79 wherein the membrane is metal oxide, glass, polyamide, polyester, polyolefin, polysaccharide, polyglycol or polyamino acid.

83. A kit for assaying for a target nucleotide sequence in a solution containing polynucleotides suspected of containing the target sequence comprising:

a. a nucleotide probe for the target sequence; and

b. a polycationic support capable of separating polynucleotides according to their charge or length.

84. (amended) A kit for assaying for a target nucleotide sequence in a solution containing polynucleotides suspected of containing the target sequence comprising:

a. a nucleotide probe for the target sequence;

polyester, polyolefin, polysaccharide, polyglycol or polyamino acid.

108. (amended) A kit according to Claim 104 wherein the fibers are a metal oxide, glass, polyamide, polyester, polyolefin, polysaccharide, polyglycol or polyamino acid.

109. (amended) A kit according to Claim 105 wherein the membrane is a metal oxide, glass, polyamide, polyester, polyolefin, polysaccharide, polyglycol or polyamino acid.

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US88/00550

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) ³

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC (4): C12Q 1/68; C07H 21/00

US CL : 435/6; 536/27

II. FIELDS SEARCHED

Classification System	Minimum Documentation Searched ⁴	
	Classification Symbols	
U.S.	435/6, 803, 810	
	436/501, 525, 526, 527, 808	
	935/78; 536/27	

Documentation Searched other than Minimum Documentation
to the Extent that such Documents are Included in the Fields Searched ⁴

COMPUTER SEARCH: APS, BIOSIS, CHEMICAL ABSTRACTS

III. DOCUMENTS CONSIDERED TO BE RELEVANT ¹⁴

Category ⁵	Citation of Document, ¹⁶ with indication, where appropriate, of the relevant passages ¹⁷	Relevant to Claim No. ¹⁸
P, Y A	US, A, 4,672,040 (JOSEPHSON) 19 June 1987. See Columns 9, lines 37-42; 10, lines 54-64; 18, lines 44-58.	1-17, 20-41 53-71, 83-99 18, 19, 42-52, 72-82, 100-109
Y A	US, A, 4,554,088 (WHITEHEAD ET AL), 19 November 1985. See Columns 9, lines 7-11 and 10, lines 14-23.	1-17, 20-41 53-71, 83-99 18, 19, 42-52, 72-82, 100-109
P, Y	US, A, 4,652,517 (SCHOLL ET AL), 24 March 1987. See Column 6, lines 1-10.	83, 84, 92, 96
P, Y	Chemical Abstracts, Volume 107, No. 23, issued 07 December 1987, J. SUMMERTON "Polynucleotide assay reagent and method" see page 304 bridging Columns 1 and 2, the abstract no. 214481;, PCT Int. Appl. WO 86 05,519.	27, 28, 30-33, 57-63, 90, 91

* Special categories of cited documents: ¹⁵

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search ¹⁹:

05 May 1988

Date of Mailing of this International Search Report ²⁰:

27 MAY 1988

International Searching Authority ²¹:

ISA/US

Signature of Authorized Officer ²²

Jeremy M. Jay

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V. OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE¹

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. Claim numbers _____, because they relate to subject matter^{1,2} not required to be searched by this Authority, namely:

2. Claim numbers _____, because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out^{1,3}, specifically:

3. Claim numbers _____, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING²

This International Searching Authority found multiple inventions in this international application as follows:

- I. Claims 1-19, drawn to a method of purification of nucleotide multimers classified in Class 536, subclass 27.

See Attachment

1. As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

- The additional search fees were accompanied by applicant's protest.

- X, No protest accompanied the payment of additional search fees.

PCT/US88/00550

Attachment To Form PCT/ISA/210, Part VI.

II. Claims 20-109 drawn to a method of hybridization and a kit for carrying out hybridization, classified in Class 435, subclass 6, for example.

The claims of these two groups are directed to different inventive concepts that are not so linked so as to form a single general inventive concept. The inventions are not linked as each group contains a separate and distinct method.